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CHARACTERISATION AND MECHANISMS OF ALTERED BODY COMPOSITION AND TISSUE WASTING IN CANCER CACHEXIA

Janice Miller

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Supervisors

Mr Richard J.E.Skipworth and Professor Stephen J. Wigmore

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DECLARATION

The thesis composition herein is my own. Where I have been a member of a research group, I have made a substantial contribution to the work, and this contribution has been asserted clearly in the text. Contributions made by others to the work are acknowledged in the text. This thesis has not been submitted for any other degree, postgraduate diploma or professional qualification.

Janice Miller

ABBREVIATIONS

ABACS	Automated Body Composition Analyser
ACC	Adenocarcinoma
ACE	Angiotensin Converting Enzyme
ACH	Acetylcholine
ACHE	Acetylcholine Esterase
ACHR	Acetylcholine Receptor
ACN	Acetonitrile
ACTB	Beta-Actin
ACTRIIB	Activin Receptor Type IIB
ACVR2	Activin Receptor Type 2
AGRN	Agrin
AICAR	5-Aminoimidazole-4-carboxamide ribonucleotide
AJCC	American Joint Committee on Cancer
AKT	Protein kinase B
ALOX15	Arachidonate 15 Lipoxygenase
AMPK	5'AMP Activated Protein Kinase
ANOVA	Analysis of Variance
APC	Adenomatous Polyposis Coli
ATG7	Autophagy Related 7
ATGL	Adipose Triglyceride Lipase
ATP	Adenosine-5'-Triphosphate
BAPEN	British Association for Parenteral and Enteral Nutrition
BAT	Brown Adipose Tissue
BIA	Bioelectrical Impedence Analysis
BMI	Body Mass Index
BNIP3	BCL2 Interacting Protein 3
BNST	British Nutrition Screening Tool
BYM338	Bimagrumab
C-26	Colon-26 Adenocarcinoma Mouse Model
CA^{2+}	Calcium
CAF	C-Terminal Agrin Fragment
CAN	Causal Networks Analysis
CASCO	Cachexia Score
CC	Cancer Cachexia
CD	Cluster of Differentiation
CDF	Cumulative Distribution Function
cDNA	complementary DNA

CEUS	Contrast Enhanced Ultrasound
CINAHL	Cumulative Index to Nursing and Allied Health Literature
Cis5FU	Cisplatin and Fluorouracil
CM	Centimetres
CNST	Canadian Nutrition Screening Tool
CONUT	Controlling Nutritional Status
cRNA	Complementary RNA
CRP	C Reactive Protein
CRUK	Cancer Research UK
CSA	Cross Sectional Area
CT	Computerised Tomography
CUZNSOD	Copper-Zinc-Superoxide Dismutase
DGC	Dystrophin Glycoprotein Complex
DICOM	Digital Imaging and Communications in Medicine
DNA	Deoxyribonucleic Acid
DOK-7	Docking Protein 7
DXA	Dual X-ray absorptiometry
ECF	Epirubicin, Cisplatin, Fluorouracil
ECM	Extracellular Membrane
ECX	Epirubicin, Cisplatin, Capecitabine
ECOG	Eastern Cooperative Oncology Group
EDC	ESPEN Diagnostic Criteria for Malnutrition
ELISA	Enzyme Linked Immunosorbent Assay
EORTC QLQ-30	European Organisation for Research and Treatment of Cancer QOL Core Questionnaire 30
ER	Oestrogen Receptor
ESPEN	European Society of Parenteral and Enteral Nutrition
EWGSOP	European Working Group on the Sarcopenia of Older People
FA	Fatty Acid
FOXO	Forkhead Box Only
FFM	Fat Free Mass
FN14	Fibroblast Growth Factor-Inducible 14
G	Grams
GADPH	Glyceraldehyde 3-phosphate Dehydrogenase
GDF	Growth / Differentiation Factor

GDNF	Glial Cell Line-Derived Neurotrophic Factor
GH	Growth Hormone
GI	Gastrointestinal
GNRI	Geriatric Nutrition Risk Index
GOJ	Gastro-oesophageal Junction
GRAFL	GDNF Family Receptor Alpha Like
GRB2	Growth Factor Receptor-Bound Protein 2
HCAP	Human Cachexia-Associated Protein
HSL	Hormone-Sensitive Lipase
VAT	Intra-abdominal adipose tissue
ICD	International Classification of Diseases
IFN	Interferon
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL	Interleukin
ITLN1	Intelectin-1
INSYST	Imperial Nutritional Screening System
JAK	Janus Associated Kinase
JNK	c-Jun NH2-Terminal Kinase
K^+	Potassium
KDA	Daltons
KG	Kilograms
KO	Knock Out
KPS	Karnofsky Performance Score
L3	3rd Lumbar vertebrae
LBM	Lean Body Mass
LC3	Microtubule-associated protein 1A/1B-light chain 3
LC/MS	Liquid Chromatography Mass Spectometry
LEP	Leptin
LEPR	Leptin Receptor
LLC	Lewis Lung Carcinoma
LO	Lower Oesophagus
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
LPL	Lipoprotein Lipase
LrP4	Low-density Lipoprotein Receptor-Related protein 4
M	Metres
mAb	Monoclonal Antibody

MABp1	Bermekimab
MAFbx	Muscle-Specific F-box (also known as Atrogin-1)
MCP-1	Monocyte Chemoattractant Protein-1
MD	Muscular Dystrophy
MDSC	Myeloid Derived Suppressor Cell
MDT	Multidisciplinary Team
MEF2C	Myocyte Enhancer Factor 2C
MENAC	Multimodal Intervention (Exercise, Nutrition and Anti-Inflammatory Medication)
MFN	Mitofusin
MG132	Carbobenzoxy-Leu-Leu-leucinal
MIC-1	Macrophage Inhibitory Cytokine 1
miRNA	Micro RNA
MRI	Magnetic Resonance Imaging
mRNA	messenger RNA
MRS	Magnetic Resonance Spectroscopy
MST	Malnutrition Screening Tool
MSTC	Malnutrition Screening Tool for Cancer
mTOR	Mammalian Target of Rapamycin
MURF-1	Muscle-Specific RING Finger-1
MuSK	Muscle-Specific Kinase
MYC	Myc-Proto-Oncogene Protein
MyHC	Myosin Heavy Chain
MYOD	Myoblast Determination Protein
MyoMiR	Muscle Specific MicroRNA
MTOR	Mammalian Target of Rapamycin
$NA2^{+}$	Sodium
NAC	Neoadjuvant chemotherapy
N-CAM	Neural Cell Adhesion Molecule
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NHS	National Health Service
NK	Natural Killer
NMJ	Neuromuscular junction
NOS	Nitric Oxide Synthases
NPY	Neuropeptide Y
NRI	Nutritional Risk Index
NRS-2002	Nutritional Risk Screening
NSCLC	Non-Small Cell Lung Cancer
NUFFE	Nutritional Form For the Elderly
OCT	Optimal Cutting Temperature Compound

OPA1	Optic Atrophy Protein
OPLS-DA	Orthogonal Projections to Latent Structures Discriminant Analysis
PACS	Picture Archiving and Communication System
PAX7	Paired Box 7
PBS	Phosphate-Buffered Saline
PCA	Principal Components Analysis
PDCD4	Programmed Cell Death 4
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol 3-kinase
PIF	Proteolysis Inducing Factor
PIK3	Phosphatidylinositol 3-kinase
PK	Protein Kinase
POLR2A	RNA Polymerase II Subunit A
PPARA	Peroxisome Proliferator-Activated Receptor A
PPARGC1A	Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-Alpha
PRDM16	PR/SET Domain 16
PS	Performance Status
PSMB2	Proteosome Subunit Beta Type-2
PTHR	Parathyroid Hormone Receptor
QoL	Quality of Life
qPCR	Quantitative Polymerase Chain Reaction
RA	Rectus Abdominis
RIE	Royal Infirmary of Edinburgh
RIN	RNA Integrity Number
ROS	Reactive Oxygen Species
RPM	Revolutions Per Minute
RNA	Ribonucleic Acid
R-NST	Renal Nutritional Screening Tool
RTx	Radiotherapy
SARMs	Selective Androgen Receptor Modulators
SAT	Subcutaneous Adipose Tissue
SATI	Subcutaneous Adipose Tissue Index
SCC	Squamous Cell Carcinoma
SD	Standard Deviation
SELP	P-Selectin
SEM	Standard Error of Mean
SGA	Subjective Global Assessment
SLC39A	Zinc transporter 11

SMAD	SMA Mothers Against Decapentaplegic
SMI	Skeletal Muscle Index
SNAQ	Short Nutritional Assessment Questionnaire
SNP	Single Nucleotide Polymorphism
SNST	Spinal Nutritional Screening Tool
SOD1	Superoxide Dismutase
SPSM	Short Portable Sarcopenia Measure
SPSS	Statistical Package for the Social Sciences
STAT	Signal Transducer and Activator of Transcription
STS	Sit to Stand
TAK1	Transforming Growth Factor Beta-Activated Kinase
TAM	Tumour Associated Macrophage
TATI	Total Adipose Tissue Index
TG	Triglyceride
TGF	Transforming Growth Factor
Th	T helper
TIL	Tumour Infiltrating Lymphocyte
TNF	Tumour Necrosis Factor
TORC1	Rapamycin Complex 1
TUG	Timed-Up-And-Go
TWEAK	Tumour Necrosis Factor-Like Weak Inducer of Apoptosis
UCP	Uncoupling Protein
UGI	Upper Gastrointestinal
UICC	Union for International Cancer Control
UK	United Kingdom
UO	Upper Oesophagus
UPP	Ubiquitin-Proteasome Pathway
UWL	Unintentional Weight Loss
VAT	Visceral Adipose Tissue
VATI	Visceral Adipose Tissue Index
VEGF	Vascular Endothelial Growth Factor
WAT	White Adipose Tissue
WHO	World Health Organisation
WL	Weight Loss
YWHAZ	Tyrosine 3 Monooxygenase/tryptophan 5-monooxygenase activation protein, Zeta

ZAG	Zinc-α2-glycoprotein (also known as AZGP1)
ZIP14	Zrt and IRT Like Protein 14
α-BTX	Alpha-Bungarotoxin
υL	Microlitres
χ^2	Chi squared
3-MinNS	3 Minute Nutrition Screening

LAY SUMMARY

Cancer cachexia (involuntary wasting syndrome) is an under-researched cause of major patient morbidity and mortality, which has no efficacious treatment or generally accepted strategy for management. Skeletal muscle and fat loss are the two markers in the diagnosis and classification of cachexia. This thesis aimed to assess molecular mediators involved in muscle and fat wasting through a variety of ways, including taking samples of muscle, fat and blood from patients with and without cancer cachexia.

Firstly, a review of tools used to diagnose unintentional weight loss was undertaken and found that no single tool was currently able to synchronously diagnose cachexia, age-related muscle loss, and simple malnutrition. Components to make up a new tool which have since been used as the subject of an editorial to inform a consensus on the definition and management of malnutrition were therefore suggested. Secondly, CT was used to investigate the effect of chemotherapy on muscle and fat wasting. All patients lost muscle mass during chemotherapy, but those who were older lost more fat, and that the type of fat that was lost was different between men and women. Thirdly, the main investigation of muscle in this study was looking at the interface between muscle and nerves. This was found to be the same between healthy individuals and patients with cancer who did and did not have cachexia, suggesting that cachexia is primarily a muscular disorder. Forthly, fat samples from patients were analysed. There was found to be large differences in

expression of genes between fat from under the skin and inside the abdomen, suggesting that fat from inside the abdomen may play a role in the cachexia process. “Intelectin-1” was identified as a possible marker of this wasting. Finally, breakdown products (metabolites) of fat wasting were identified from the blood of patients with weight loss, suggesting this may be of use as a test to monitor the effects of future anti-cachexia treatments.

It is hoped that results from this study will identify potential therapeutic targets and help plan future studies.

ABSTRACT

Cancer cachexia has been defined as a multifactorial syndrome characterised by an ongoing loss of skeletal muscle that cannot be fully reversed by conventional nutritional support. Cachexia affects most patients with advanced cancer and is associated with reductions in treatment tolerance, response to therapy, quality of life, and survival. Thus, amelioration of cachexia would improve both quality of life and clinical outcome. However, the aetiology of cachexia is poorly understood, and there are no agreed diagnostic biomarkers or management strategy for patients with cancer cachexia.

Recent advances in the field of cachexia research include the development of diagnostic criteria for cachexia, as well as computed tomography (CT) body composition analysis software, making the ability to detect clinically significant muscle wasting in obese patients in particular more accurate. Although muscle loss appears to be the most important and physiologically relevant event in cachexia, the importance of fat wasting is less understood. During cachexia, different adipose depots around the body demonstrate differential rates of wasting. Furthermore, recent studies from animal models have suggested that adipose tissue may be a key driver of muscle wasting through fat-muscle crosstalk. However, human studies in this area are lacking. The molecular mechanisms driving muscle loss in humans are also poorly understood, and the relationships between muscle and fat wasting, functional impairment and reduced survival are largely unknown. The prognostic significance of adipose

wasting and investigations in tissue cross-talk therefore are now becoming more important whilst the quest for a cachexia related biomarker remains at the fore.

The main aim of this thesis was to investigate specific mediators, mechanisms and biomarkers of cachexia in robustly phenotyped patients with upper gastrointestinal cancer (UGI) in whom cachexia is known to be prevalent.

This thesis is comprised of several projects designed to investigate various areas of cachexia pathophysiology, diagnosis and staging. In order to recruit patients to clinical trials, drive cachexia research and identify those who would benefit from early intervention, it is important to understand how to screen and diagnose patients with cachexia. Many patients present to clinicians with unintentional weight loss (UWL). This can occur in patients with cachexia, sarcopenia and malnutrition. With increasing rates of obesity worldwide, as well as an ageing population, differentiating causes of UWL is difficult. Firstly therefore, in order to investigate the feasibility of screening for UWL a systematic review was undertaken in chapter 3 to determine which screening tools were able to assess cachexia, sarcopenia and malnutrition according to the consensus definitions for each. Each tool was judged against a reference method and psychometric evaluation carried out. No one tool was able to assess all three conditions simultaneously, and out of the 22 tools assessed, only 3 had been validated against the gold standard of CT cross-sectional imaging. Thus, the development of a novel tool that encompasses the

consensus definition criteria and directs clinicians towards the underlying diagnosis would likely improve detection and outcomes.

Secondly, building upon screening and methods for diagnosing low muscularity, chapter 4 uses CT body composition analysis to determine any age and sex-related variations in patients with UGI cancer. CT-based cut-offs for determining low skeletal muscle volume are sex and body mass index (BMI) specific and have been driven in order to predict mortality in these patients. As discussed above, the prevalence of obesity is increasing and the population is ageing therefore, many patients may be sarcopenic at diagnosis, making the assessment of clinically significant muscle wasting difficult. A retrospective, observational study was carried out on patients who had undergone potentially curative oncological and surgical treatment for oesophageal cancer. Analysis of both staging and post neoadjuvant chemotherapy (NAC) CT was performed in order to assess baseline characteristics and dynamic changes in body composition. Males had higher baseline muscle and visceral fat volume whereas females had higher subcutaneous fat volume. Patients of all ages and both sexes lost muscle volume though there was no difference in rates of wasting between groups. Older patients and females lost significantly more total fat during chemotherapy. This chapter therefore highlights the need for further investigation to define differences in adipose depots during cancer progression and their prognostic value.

Chapter 5 showcases the main biological assessment of cancer associated muscle wasting in this thesis. As shown in chapter 4 all patients demonstrated some evidence of muscle wasting. A potential mechanism of this was therefore investigated further by looking at the role of the neuromuscular junction (NMJ). The NMJ provides the link between myelinated motor nerves and skeletal muscle. Very little is known about the structure of the NMJ in human health or in disease. Experimental denervation is a recognised model for studying muscle wasting *in vivo*, and as a result experimental evidence for the role of the NMJ in cachexia is dependent upon animal models. Recent data, however have shown that rodent and human NMJs are markedly different. NMJ morph, an imageJ-based package was used for morphometric analysis of the NMJ in UGI cancer patients with or without cachexia and non-cancer controls. No significant differences were found between groups in any of the major pre- or post-synaptic variables measured suggesting that the NMJ remains structurally intact in cancer cachexia, and thus, the denervation of skeletal muscle is not a major driver of the disease.

Whilst it is recognised that muscle mass plays a significant role in the syndrome of cancer cachexia, as shown in chapter 4 through body composition analysis the importance of fat wasting and the effect of metabolic mediators on fat volume requires attention. In murine tumour models, loss of fat volume may predate the loss of muscle volume. Fatty acids, leptines, cytokines and other adipokines may cause lipotoxic effects in skeletal muscle.

Adipokines have been reported to induce insulin resistance, impair muscle development, alter muscle lipid amino acid metabolism and modify signalling thus affecting skeletal muscle volume. Clinical studies have shown that adipokines from murine models are also measurable in patients with cancer cachexia. In chapter 6 through the use of transcriptomics, subcutaneous (SAT) and visceral adipose tissue (VAT) depots were analysed from UGI cancer patients with and without cachexia and healthy controls to elucidate the biochemistry of fat wasting in cancer cachexia. Over 2000 genes differed between cachexia VAT and SAT. The gene that showed the largest difference in expression between cancer VAT and control was Intelectin-1 (ITLN1), a novel adipocytokine. Genes involving inflammation were upregulated in cancer whereas genes involved in energy metabolism and fat browning were down regulated. VAT, therefore, may be a target for therapeutic manipulation in cancer. Further investigation is required in to the role of Intelectin-1 as a biomarker in cachexia.

Finally, in previous searches for biomarkers of cancer, likely responsiveness to treatment and the presence of cachexia, plasma has been used as a readily available biofluid for investigation. However, no robust cachexia biomarker has been found. Although as work continues it seems that individual biomarker targets should be replaced by an array of markers. Chapter 7 used liquid chromatography mass spectrometry (LC/MS)-based metabolomics to investigate the metabolic profile of weight loss from plasma samples taken at

the time of anaesthesia from patients under-going UGI resectional surgery. This showed two distinct profiles based on percentage weight loss in accordance with the consensus definition. There were 40 metabolites associated with cachexia with six of those being highly discriminative of weight loss. Specifically, a combination profile of LysoPC 18.2, Hexadecanoic acid, Octadecanoic acid, Phenylalanine and LysoPC 16.1 showed close correlation for eight weight-losing samples (≥5% weight loss) and nine weight stable samples (<5% weight loss). In particular ,many of the metabolites discovered were involved in lipid metabolism, lending credence again to the importance of understanding adipose wasting in cachexia.

In summary, the role of adipose wasting as investigated through imaging and biochemical results has been shown to be important in the aetiology of cancer cachexia. It has been demonstrated that there is currently no adequate way to screen for conditions which present with UWL and that the adaptation of a tool in order to do this would drive further research and the content of complex interventions. Previously published CT-derived cut points are BMI and sex specific, however, it has been shown that there is a growing need to develop these in order to define patients by age also. In doing so this would define stricter criteria for clinical trials and lead to improved end points. Potential novel biomarkers of lipid wasting have been discovered in this thesis. ITLN1 which has corresponded with weight loss previously in other groups warrants further investigation as it may be a target for future therapeutic manipulation. Those

biomarkers discovered in the metabolomics study show that it is possible to separate patients based on weight loss alone. Although this was a pilot study after further investigation and development, biomarkers of lipid wasting may be useful as inclusion criteria or outcome measures in clinical trials. The discoveries of the lack of fat browning and the stability of the NMJ in cachectic patients also importantly highlights the need for patient rather than animal based research.

CHAPTER 1
INTRODUCTION

1.1 Overview

This thesis represents a comprehensive, and importantly human, approach to the unmet clinical need afforded by cancer cachexia. By performing body composition analysis and biochemical studies on plasma, muscle and adipose tissue, the project aims to provide a broad and wide-reaching analysis of cachectic markers in robustly phenotyped patients. The development of successful treatments for cancer cachexia would be a major advancement in the treatment of virtually all tumour types as almost all patients with advanced disease demonstrate weight loss. Treatments to decelerate or reverse muscle loss would improve both quantity and quality of life. The following introductory chapter provides an overview of cancer cachexia with a particular focus on novel mediators that have been shown to be of relevance in human disease. Previously trialled therapies are discussed alongside those that offer promise for the future.

Cachexia comes from two Greek words: “Kakos” meaning bad and “hexis” meaning condition (1). It has long been recognised as a side effect of many chronic conditions such as renal, heart and liver failure, and chronic lung disease; however, it is most prevalent in patients suffering from cancer (2). It is estimated that half of all cancer patients experience weight loss, and one third lose more than 5% of their body weight, with this value increasing to 86% in the last 1 to 2 weeks of life (3,4). This weight loss is due to depletion of both

adipose tissue and skeletal muscle mass (5). Symptoms of cachexia include unintentional weight loss (UWL), anorexia, inflammation and muscle wasting resulting in a decreased quality of life (6). This muscle wasting can involve the chest, diaphragm and cardiac muscle, and therefore many patients die of cardiac or respiratory failure (7,8). Cachexia has adverse effects on treatment and outcomes and is considered the immediate cause of death in a large proportion of cancer patients (6) (figure 1). It is therefore essential to apply a systemic approach to its investigation and management. It is also important to recognise the effect the tumour has on the disease, and thus the complex interaction between it and the host.

Cachexia is particularly prevalent in patients with UGI cancer (>80% patients affected), including oesophageal and pancreatic cancer (6,9), two tumour types highlighted by the research strategy of Cancer Research UK (CRUK) (10). Cancer cachexia remains an unmet clinical need, because even in the current era of multimodal therapies (including surgical resection with neoadjuvant/adjuvant oncological treatments), patients with UGI cancer are still likely to develop disease recurrence and die, with median survival times of approximately 2 years (5-year survival rates for oesophageal and pancreatic cancer are 15% and 5%, respectively) (11). Therefore, holistic treatment strategies for patients with unresectable or recurrent disease are a priority.

Muscle wasting in cancer is multifactorial and complex. It is underpinned by hypercatabolism, a net loss of energy, and systemic inflammation (12) (figure

2). Classical understanding of muscle atrophy has focused on the imbalance between protein synthesis and degradation; however, new evidence has also raised the issues of contribution of apoptosis and impaired renewal. Signalling pathways that have been investigated previously include those involved in systemic inflammation, TGF-family signalling and autophagy (13). Many possible mediators continue to be discovered, and those that lend themselves as therapeutic targets are discussed in more detail later in this introduction. Although loss of muscle mass is a common feature of cancer cachexia, differences in the underlying mechanisms have been reported between different experimental models and human tumour types (14,15). This diversity leads to challenges in the development of effective therapeutic management strategies, as well as difficulties in the design of clinical trials.

Figure 1 Clinical features of cancer cachexia

Cancer cachexia leads to a range of symptoms including changes in body composition and psychological distress ultimately leading to poor surgical and oncological outcomes.

RTx - radiotherapy

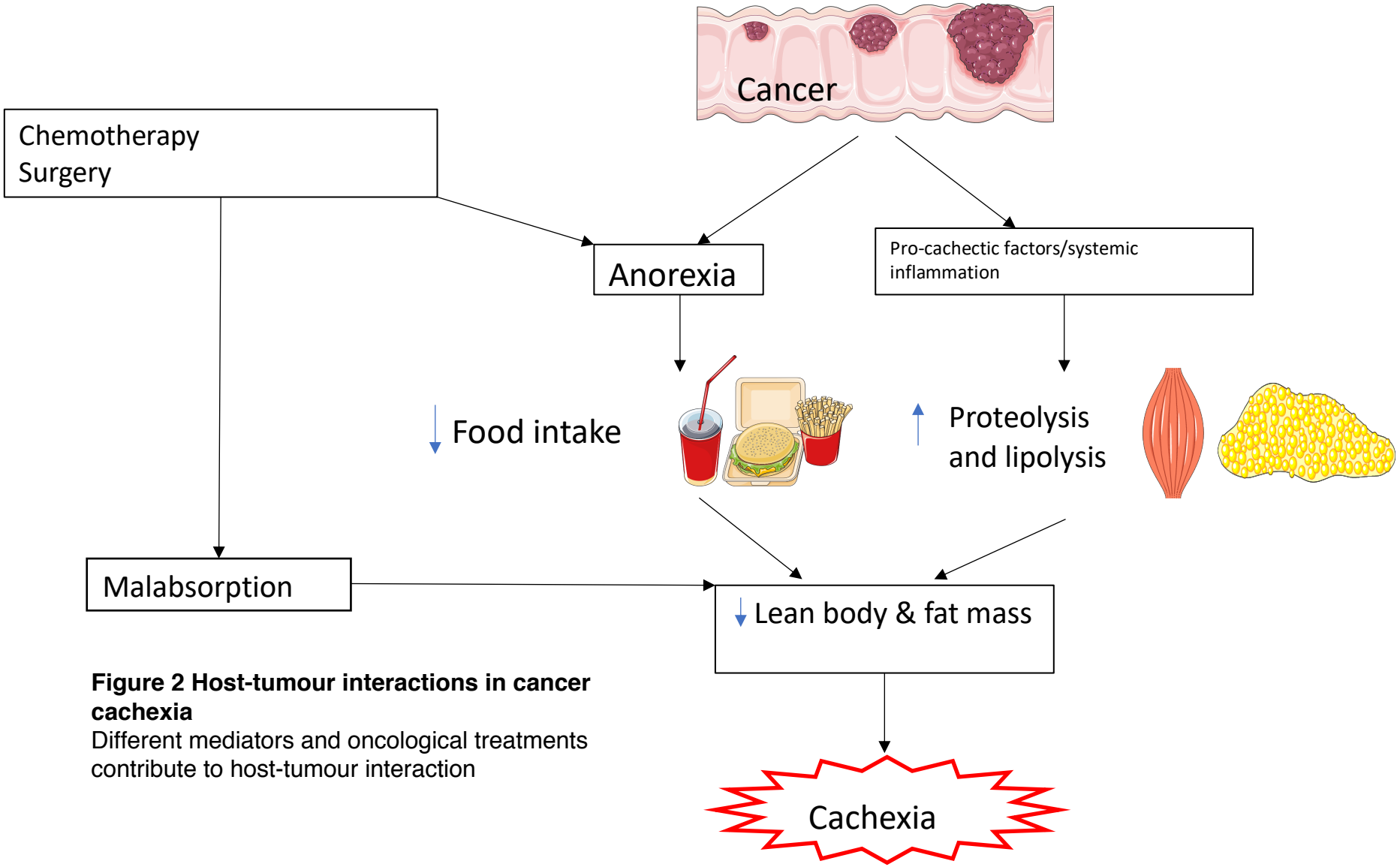


Figure 2 Host-tumour interactions in cancer cachexia
Different mediators and oncological treatments contribute to host-tumour interaction

The previous section has offered a basic overview of cancer cachexia and has very simply described some of the underlying mechanisms. In order to understand the clinical relevance of cachexia we must firstly consider the importance of normal muscle structure and function to then understand why abnormalities in it are important in the disease process.

1.2 Muscle structure and function

Skeletal muscle has a complex structure and function. The healthy human body is composed of approximately 40% muscle tissue, which accounts for 30-45% of whole body protein turnover (16). Skeletal muscle is highly adaptable, being able to undergo hypertrophy or wasting in response to various stimuli. Skeletal muscle differentiation is complete by the time of birth meaning that differentiated myofibres are unable to divide or grow new muscle tissue. Muscle tissue instead contains undifferentiated stem cells called satellite cells. These cells are able to respond to stimuli and produce daughter cells that differentiate into myoblasts which can then fuse together or with existing myofibres to enlarge or repair them (17). The opposite can also occur meaning muscle can atrophy in response to opposing stimuli or an increase in protein degradation signaling (18). As well as its primary role in movement of the body, muscle also serves as an amino acid store. It can be broken down to provide glutamine and amino acids as energy stores during the acute phase response to injury. It also plays a role in thermoregulation, insulin resistance, and glucose metabolism (19).

All muscle types in animals and humans have a common basic structure including actin and myosin protein filaments that make up the thick and thin muscle filaments. Myosin is the larger filament and is the force-generating form. It consists of two large polypeptide heavy chains and 4 small light chains. Actin is a 42kDa globular protein found in all eukaryotic cells and is a major component of the cytoskeleton. In muscle it makes up the thin filament along with troponin and tropomyosin. In skeletal muscle the thick and thin filaments are arranged into bundles called myofibrils, that in turn are grouped together into muscle fibres or cells.

Myofibrils are an aggregation of filaments that run parallel to the long axis of the muscle fibre. Each fibre contains several hundred myofibrils, making up the largest part of the muscle fibre volume. Myofibrils are surrounded by sarcoplasmic reticulum which acts to release Ca^{2+} in response to the muscle action potential – the excitation contraction coupling process. The whole myofibre is bound by the sarcolemma which contains myofibrils, endoplasmic reticulum, nuclei and mitochondria (20). Mitochondria in the I band (figure 3), in particular, have a highly convoluted structure in order to fit into the restricted space available between myofibrils. The number of mitochondria varies between muscle fibres. Those relying on aerobic glycolysis (slow twitch or type 1 fibres) are rich in mitochondria, whereas those who have a higher utilization of anaerobic energy (fast twitch or type 2 fibres) have fewer (21).

Muscle contraction occurs when the myosin heads interact with the actin of thin filaments. The movement of muscle occurs as the fibre and their myofibril subunits shorten. When the skeletal muscle shortens, thick and thin fibres of the sarcomere move past each other in a sliding filament mechanism (22). This mechanism involves the formation of a cross bridge between thick and thin filaments. The myosin head binds to actin, the cross bridge moves and is then dissociated, allowing the process to be repeated. The energy is provided by Adenosine triphosphate (ATP) hydrolysis at the ATPase on the myosin heavy chain. Tropomyosin regulates this process by blocking the myosin binding sites on actin. Calcium (Ca^{2+}) acts on troponin, thus moving the tropomyosin to expose the binding sites (23).

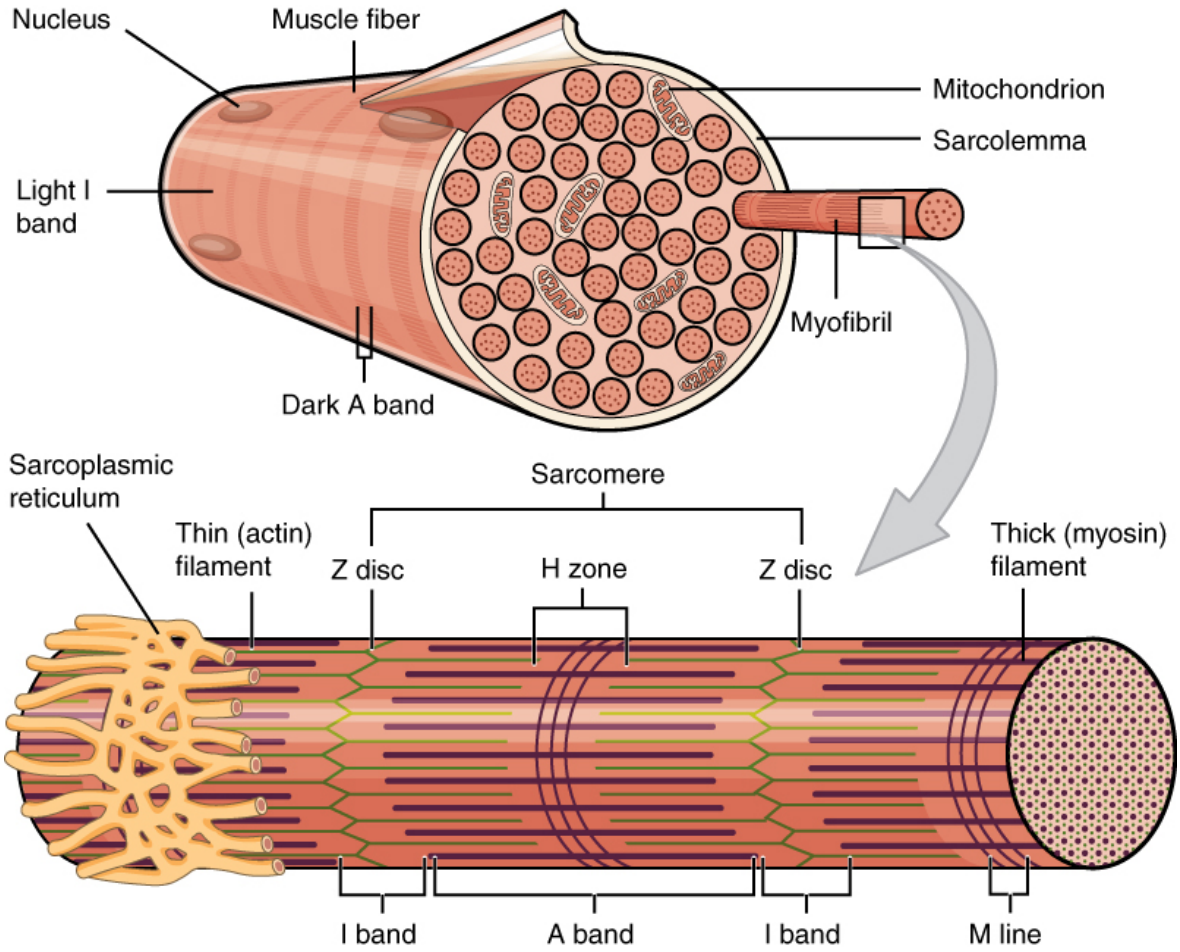


Figure 3 Muscle structure (24)

The structure of the myofibril is made up of repeating sarcomeres, the centre of which is called the M line. This is a protein disc to which parallel thick filaments are attached known as the A band. Inter-linking with the thick filaments are overlapping thin filaments. The area of no overlap is known as the I band, and their attachment at either end of the sarcomere to a protein disc is known as the Z line.

1.3 What is cachexia?

So why are abnormalities in the normal structure and function of muscle relevant to patients with cachexia? In order to understand the answer to this question we must first define what cachexia is and appreciate the scale of the problem.

1.3.1 Definition of cancer cachexia

Historically, cachexia was defined as loss of weight (involuntary weight loss >10%) (25). More recent, definitions, however recognise that weight loss may be underestimated in overweight or obese patients or in patients who have gained weight because of oedema or a growing tumour mass (26). The previous image of the “wasted cachectic patient” is not usually seen due to the growing obesity epidemic. Therefore, many cachectic patients may have a normal or raised body mass index (BMI) (27). Due to this increasing complexity in diagnosis, a panel of experts agreed on a consensus definition. The international consensus definition states “Cancer cachexia is a multifactorial syndrome defined by an ongoing loss of skeletal muscle mass (with or without loss of fat mass) that cannot be fully reversed by conventional nutritional support and leads to progressive functional impairment” (28). The agreed diagnostic criterion for cachexia were:

- Weight loss >5% over the past 6 months (in absence of simple starvation); or
- BMI <20 and any degree of weight loss >2%; or

- Appendicular skeletal muscle index consistent with sarcopenia (males <7.26 kg/m^2; females <5.45 kg/m^2) and any degree of weight loss >2% (28)

The diagnosis of cancer cachexia requires the fulfilment of one of the three criteria. The consensus definition has previously been validated showing that those patients who full fill the cachexia criteria have shorter survival times than those who are not defined as being cachectic. However, it does not aid in estimating the prognosis of the cachectic patient (29). Factors such as quality of life (QoL) and physical function are also important to consider. Previous studies have compared this current definition with existing definitions, most notably that by Evans et al in 2008 (30). The Evans et al. definition included defining decreased muscle strength, anorexia, symptoms of fatigue or biochemical abnormalities. It was found that the Evans criteria did in fact show greater discrimination with regards to overall survival (31). The current consensus definition also uses Dual x-ray absorptiometry (DXA) cut points which although still in existence have now been superseded by CT imaging and BMI-stratified criteria (32). Perhaps therefore the 2011 definition requires revision incorporating more biochemical and functional measures, as well as CT analysis of body composition?

1.3.2 Staging of cachexia

The assessment of severity of cachexia is complex. The consensus group also suggested a method for staging severity (28). Cachexia can be seen on a spectrum from pre-cachexia to cachexia to refractory cachexia (figure 4).

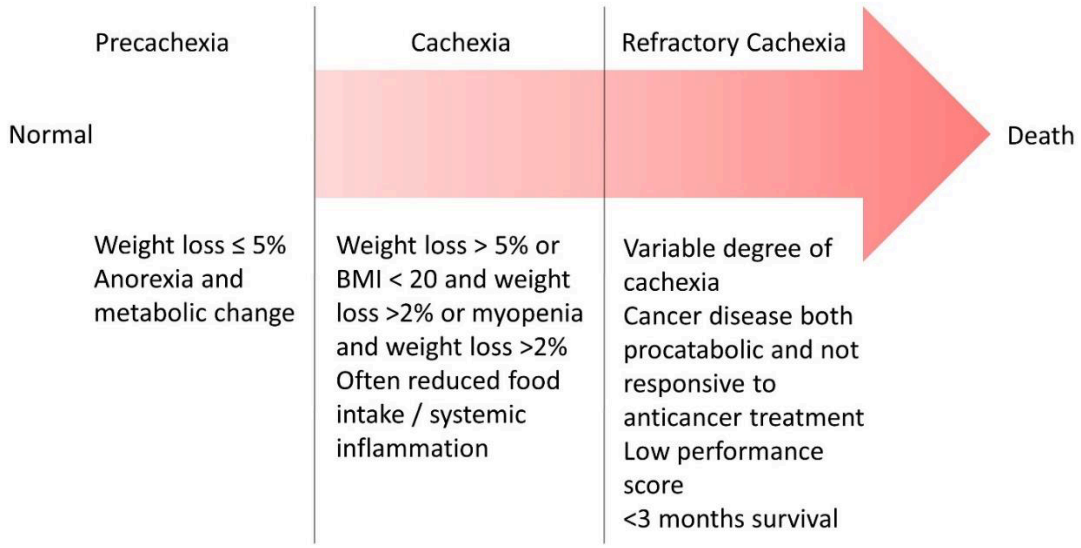


Figure 4 Staging of cachexia [adapted from (28)]

Progression of cachexia throughout the disease process. Patients who are pre-cachectic would benefit the most from intervention but they pose the largest diagnostic challenge.

Five domains were suggested to be assessed including food intake, biochemical derangements, functional implications and assessment of adipose and muscle mass (28). These stages of cachexia were suggested to be of use for prognostication and for discussion with patients, but their use in diagnosis remains difficult due to the subtlety of observable differences between pre-cachexia and cachexia, in particular. At the severe end of the scale, the accurate diagnosis of refractory cachexia is reliant on monitoring the patient's overall clinical condition, and the specific response of their tumour to treatment. A study in cancer patients investigated this three level staging system with respect to QoL, morbidity and mortality. It found that those in the pre-cachectic and cachectic group behaved in the same way (33). A further study assessing weight loss at diagnosis suggested a survival benefit to those who were pre-cachectic, but this was only apparent one year after diagnosis (34). More recent developments have seen the introduction of "The Cachexia SCOre" also known as the CASCO (35). This screening tool incorporates five components including weight loss and body composition, metabolic derangements, physical performance, QoL and anorexia. It was developed to aid in staging cachexia and is discussed in more detail later on.

In order to help clinicians with recognising and diagnosing cachexia earlier and to facilitate research, a single universal definition would be helpful. In 2017 the European Society of Clinical Nutrition and Metabolism (ESPEN) recommended that all patients with cancer should be screened for nutritional

risk and that this should include an assessment of body composition and inflammatory markers (36). The best way in which to do this risk assessment remains up for debate. Multimodal interventions should then be instigated, focusing on increasing nutritional intake and exercise and decreasing inflammation.

The main aim of all of these definitions and screening tools is to identify patients with pre-cachexia in order to instigate prophylactic interventions. The lack of strict diagnostic criteria also hinders clinical trials and leads to difficulties in comparison between studies due to the use of multiple different inclusion criteria. Previous cachexia trials have seen high attrition rates, and therefore use of the staging criteria would avoid the recruitment of patients with refractory cachexia into clinical trials and aid biomarker development (37). Much of this thesis therefore is targeted at patients early on in their disease process to identify and investigate those who may benefit from future therapeutic interventions.

1.4 Epidemiology of cancer cachexia and impact of tumour type

As discussed above, definitions of cachexia used in research vary by research group, and this variability leads to an impact on reported prevalence. One of the most well-known studies to investigate the various definitions reported that the proportion of palliative care cancer patients suffering from cachexia ranged

from 12 to 85% depending on the definition used (38). The incidence of weight loss upon diagnosis has been shown to vary greatly depending upon the site of the tumour. Arguably the biggest study to investigate this was the Eastern Cooperative Oncology Group (ECOG) chemotherapy trial (6). This study involved over 3000 patients and concluded that the greatest incidence of weight loss could be found in patients who had solid tumours i.e. Pancreatic, gastric, lung, colorectal, and head and neck. Cachexia was most prevalent in those with pancreatic and gastric cancer with around 85% of them showing signs of weight loss compared to approximately 30% of patients with breast cancer or leukaemia (Table 1). One third had more than 10% weight loss, although evidence of any weight loss, no matter how minor, was associated with poorer prognosis. Patients with weight loss who were considered to have a better performance status or an early tumour stage had better outcomes suggesting that interventions to treat cachexia should be considered early on (6).

Cancer type	Weight loss in the previous 6 months %			Percentage of patients with any weight loss	Percentage of patients with no weight loss
	0-5	5-10	>10		
Gastric	20	29	38	87	13
Pancreatic	29	28	26	83	17
Non-small cell lung cancer	25	21	15	61	39
Small cell lung cancer	23	20	14	57	43
Prostate cancer	28	18	10	56	44
Colon cancer	26	14	14	54	46
Unfavourable Non-Hodgkin's lymphoma	20	13	15	48	52
Acute non-lymphocytic leukaemia	27	8	4	39	61
Breast	22	8	6	36	64
Favourable non-Hodgkin's lymphoma	14	8	10	32	68

Table 1 Cancer types with the highest rates of cachexia (6). Patients with solid tumour types in particular demonstrated a higher prevalence of cachexia as defined by percentage weight loss.

Despite the use of different definitions there is thought to be an overall prevalence of cachexia in advanced cancer ranging from 60 to 80%, with that rising to 86% in the last few weeks of life (3,4). Cancer cachexia is thought to contribute to 30% of cancer deaths (3). Furthermore, the true percentage of cachexia related deaths may be being masked by the presence of obesity with sarcopenic obesity (discussed in more detail later), being shown to be an independent adverse prognosticator (27).

Of particular note is the aggressive nature of pancreatic and gastric cancers. The prevalence of cachexia has been investigated in 8451 cancer patients using four different definitions (39). Namely 1: The International Classification of Diseases (ICD) definition, 2: ICD definition, anorexia, abnormal weight loss or feeding difficulties, 3: Prescription for the use of 'anti-cachectic' drugs such as megestrol, acetate, oxandrolone, somatropin or dronabinol and 4: ≥5% weight loss. There were varying prevalence rates according to the different definitions (2.5, 5.5, 6.4 and 14.7% respectively) (39). Although this was a large study, it highlighted problems with the ICD definition as only 3.9% of patients with pancreatic cancer were defined as being cachectic, which is in contrast to most other studies. Even when comparing the consensus definition against two other classifications of cachexia - the SCRINIO definition (based on >10% weight loss and either anorexia, fatigue or early satiation) and the three factor definition (weight loss >10%, low food intake and raised C-reactive

protein [CRP]) - there was again wide variation in prevalence rates from 12 to 85% (39).

Aside from UGI malignancy, lung, and head and neck cancer patients are also well known to suffer drastic weight loss. Between 59 to 76% of patients with lung cancer are reported to have weight loss at presentation compared with >30% of head and neck cancer patients (40,41), although the latter group are likely to have difficulties with dysphagia and therefore maybe suffering from malnutrition rather than cachexia.

The reasons why some cancers are more strongly associated with cachexia than others are not clear. It has been hypothesised that some cancers are more likely to present earlier in the disease process than others and therefore are diagnosed before any weight loss occurs (42). Alternative suggestions have involved the acute phase response and hypermetabolism being more common in certain cancers, therefore leading to a more rapid loss of skeletal muscle (43). There is also variation of the presentation of cachexia between patients with the same type of cancer e.g. up to 85% of patients with pancreatic cancer will present with cachexia but 15% will not, possibly due to a differing phenotype or genotype (44). Candidate gene studies have shown that single nucleotide polymorphisms (SNPs) in the P-selectin, ACVR2B, ACE, LEPR and TNF genes were associated with low muscle mass in patients with cancer (45). What is clear is the need for a widely agreed upon definition in cachexia

research. With some studies published after the consensus definition in 2011 still using different diagnostic criteria or cut-off values, it makes the comparison of cancer types and patient groups very difficult. Body composition analysis techniques offer the ability to standardise the diagnosis of cachexia, allowing for accurate quantification of muscle and adipose tissue even in obese patients.

1.5 Mechanisms underlying muscle wasting in cachexia

Now we have an understanding of normal muscle function and why cachexia poses an important problem to cancer patients. Next the mechanisms underlying its pathophysiology will be discussed before going onto describe why this is relevant for clinicians.

1.5.1 The neuromuscular junction

The main focus of muscle-specific work in this thesis relates to the role of the neuromuscular junction (NMJ) in cachexia, and so this will be discussed in more detail. Skeletal muscle innervation culminates in the formation of the NMJ. It is the point where the peripheral nervous system and skeletal muscle fibres interact, and is therefore of great functional importance in locomotion and respiration.

Neuromuscular transmission is complex, and deficits in neurotransmission can lead to muscle weakness, wasting and paralysis. The structure and function of the NMJ has been well characterized in animal models, but less so in humans. Although many concepts derived from rodent NMJs have been validated by the identification of the same genes in humans, research performed in animals does not necessarily translate to patients with neuromuscular disorders. Indeed, a recent study characterizing the human NMJ it was shown to be inherently different from the mouse NMJ. The previous assumption that human NMJs are larger and more unstable than those of the rodent has been shown to be inaccurate (46). Therefore, there is a requirement for completely human-based studies to investigate the molecular mechanisms and therapeutic possibilities for treating NMJ-related disease.

1.5.1.1 NMJ structure and function

Lower motor neurones are organised functionally into motor units. The motor unit consists of a single alpha motor neuron and all of the muscle fibres it innervates, and it is the smallest functional component of the neuromuscular system. The lower motor neuron cell body is located in the ventral horn of the spinal cord, whilst the terminal part of its axon branches to form the pre-synaptic component of the NMJ. All of the muscle fibres within a single motor unit have the same characteristics and are all activated together (47). Motor units have a spatial distribution of several centimetres in length and 5-10mm in depth in larger limb muscles. The pattern of motor unit organisation is initially

fluid during polyneural innervation of muscle fibres. During the process of synapse elimination, there is a gradual reduction in the number of axonal inputs onto each muscle fibre (47). This process continues until each muscle fibre is innervated by a single motor nerve axon (mono-innervation) (figure 5).

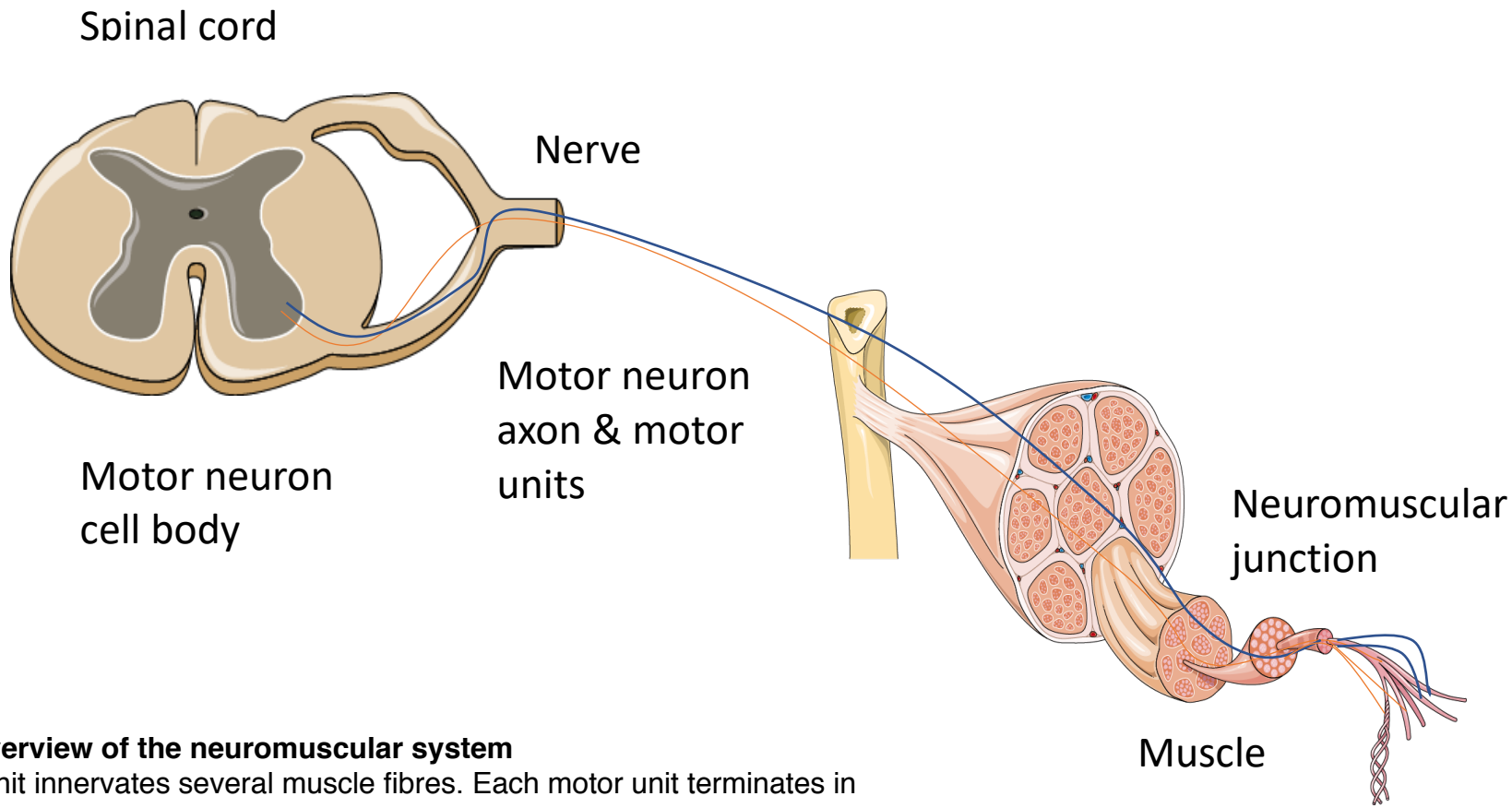


Figure 5 Overview of the neuromuscular system
One motor unit innervates several muscle fibres. Each motor unit terminates in the neuromuscular junction; the connection between the nervous and skeletal muscle systems.

NMJs develop at specific locations in muscles. They are located together in a narrow, central region of the muscle termed the ‘endplate band’. Each NMJ connects a single motor axon to a single multinucleated muscle fibre. The distal ramifications of each axon are known as nerve terminals or synaptic boutons. These boutons synapse with the motor endplate on the surface of the muscle fibre (48) (figure 6).

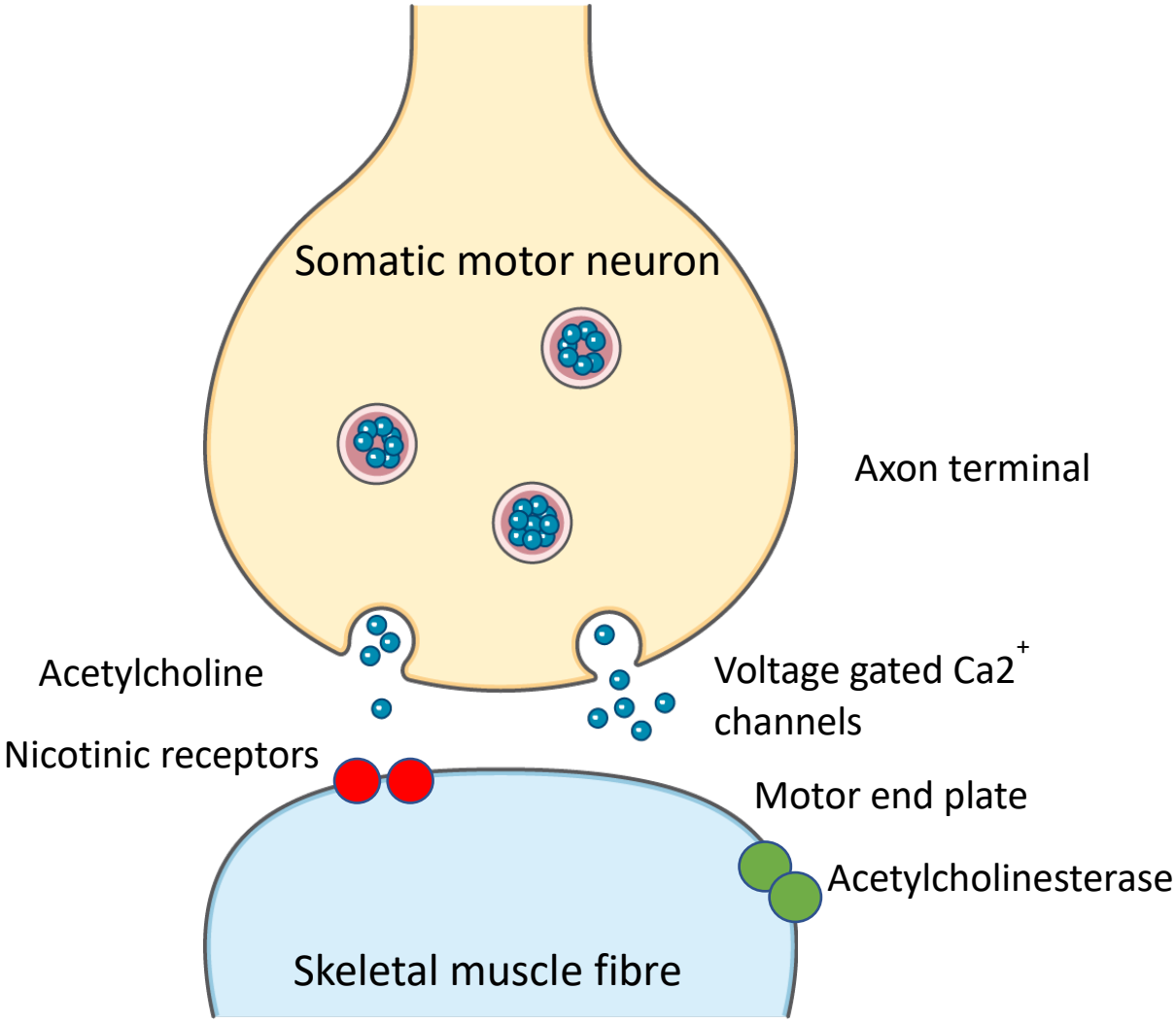


Figure 6 Events involved in the transmission of an action potential at the neuromuscular junction. Tens of millions of ligand-gated acetylcholine receptors (AChRs) and voltage-gated sodium channels are densely organised in the post synaptic junctional folds of the motor endplate. Effective synaptic transmission results in large endplate potentials in muscle fibres, evoked by nerve excitation. This is mediated by calcium-dependent exocytosis of synaptic vesicles from motor nerve terminals. As an action potential reaches the axon terminal, it induces the opening of voltage-gated Ca^{2+} channels on the presynaptic nerve membrane. This allows an influx of Ca^{2+}, which causes synaptic vesicles to fuse with the presynaptic membrane, releasing the neurotransmitter acetylcholine (ACh) into the synaptic cleft. ACh then diffuses and binds to AChRs on the postsynaptic muscle fibre membrane. In doing so the AChRs become permeable to Sodium (Na^+) and Potassium (K^+) which opens the associated voltage-gated Na^+ channels on the muscle membrane. This initiates an action potential causing Ca^{2+} to be released from the sarcoplasmic reticulum into the cytosol, and in doing so, leads to muscle contraction.

About three times as much neurotransmitter is released over the amount required to trigger an action potential into a muscle twitch. The action of ACh is stopped by the enzyme acetylcholinesterase (AChE), which is found in the synaptic basal lamina between the presynaptic terminal and the postsynaptic muscle fibre. Acetylcholinesterase that encases muscle fibres rapidly inactivates ACh released from the presynaptic membrane. Therefore, the concentration of ACh in the synaptic cleft falls rapidly, stopping neurotransmission (48).

1.5.1.2 Evidence for the role of the NMJ in cachexia

A previous study has shown abnormalities in both the sarcolemma and the associated basal lamina of muscles isolated from colon carcinoma C26-bearing mice (a recognised model of cachexia) and cancer patients. Sarcolemma; abnormalities observed in cachectic muscles were associated with myofibre damage that was similar to that observed in Duchenne Muscular Dystrophy (DMD) patients, although less severe. The muscle analysis of C26-bearing mice also identified common DGC alterations between Muscular Dystrophies (MDs) and cancer cachexia, including reduced dystrophin levels, compensatory up regulation of utrophin, and hyperglycosylation of β-dystroglycan and β–sarcoglycan. These changes were also observed in mice bearing Lewis lung carcinoma (LLC), gastrointestinal and gastro-oesophageal adenocarcinomas (49).

Central nuclei are considered to be a sign of skeletal muscle fibre regeneration and therefore a sign of myopathy. Central myonuclei have previously been observed in both pre-cachectic patients and animal models of cancer, in contrast with the reduced regenerative potential of cachectic muscles. Mononuclei have recently been further characterised in pre-cachectic and cachectic cancer patients, as well as in C26 tumour-bearing mice (50). *Rectus abdominis* and *Tibialis Anterior* were examined. Non-peripheral myonuclei were observed in two different populations – central nuclei, and nuclei that were not central nor subsarcolemmal and therefore "displaced". Both these types of nuclei were identified in all types of muscle fibres; however, displaced nuclei were the only ones that varied between cachectic and control muscles. They were also observed in the absence of muscle regeneration markers e.g. embryonic or fetal myosin. Longitudinal sections revealed clustering of displaced myonuclei upon denervation, and that these clusters were increased in cachectic patients. The increase in clusters was also associated with the upregulation of N-CAM, another marker of denervation (50). This suggests that the presence of displaced nuclei is consistent with motor neuron loss, indicating that change in NMJ state may be a cause of cachexia. Therefore, this could imply that further investigation of denervation, rather than myofibre damage and regeneration in cachexia, might be of relevance.

The role of the NMJ in cardiac cachexia has been studied in rats induced with heart failure by monocrotaline. The *soleus* muscle was harvested and used for

analysis. Ultrastructural and morphological analysis revealed no difference between cases and controls. Confocal microscope analysis showed disaggregation of the nAChRs. Aggregates of nAChRs were distributed as continuous branches that were elongated, smooth and fluorescent, forming continuous channels along the muscle fibre. In the heart failure group, nAChRs presented a spot or island pattern characterised by discontinuous fluorescent areas in the NMJs, suggesting that they had undergone potential remodelling. Investigation of nAChR subunit gene expression showed upregulation of the alpha1 and epsilon subunits of nicotinic AChR in the heart failure group (51).

Oxidative stress is thought to play a role in the development of cancer cachexia by inducing a greater expression of the ubiquitin-proteosome pathway (UPP) in skeletal muscle, thus contributing to protein degradation. Mitochondrial dysfunction may lead to altered calcium buffering, less ATP and more reactive oxygen species production (ROS). The impaired buffering of Ca^{2+} leads to an increased concentration of cytosolic Ca^{2+}, which may activate calpains. These interact with rapysn to disrupt AChR clusters (52). A direct correlation between increased oxidative stress and neuromuscular function was found in mice lacking the antioxidant enzyme Cu/Zn/superoxide dismutase (CuZnSOD). These mice showed high oxidative damage and rapid onset of muscle wasting. NMJ morphology was altered in 18-month-old $Sod1^{-/-}$ mice and was comparable to older (30-month) wild type mice. Denervated NMJs and fragmented AChRs were observed in young $Sod1^{-/-}$ mice, and they had

noticeably reduced muscular contractile force (52). This suggests that NMJ degeneration may be due to oxidative stress, produced by mitochondrial dysfunction.

1.5.2 Muscle specific mediators of cachexia

There are many mediators of pathways leading to muscle wasting. Most of these pathways are involved in protein degradation. Some act directly within or on muscle cells whilst others act as local paracrine mediators.

1.5.2.1 Protein metabolism

As discussed, skeletal muscle is a highly adaptive tissue, capable of altering its size, capacity and function in response to various stimuli. Proteolysis is controlled by two important cellular degradation systems namely the Ubiquitin Proteasome Pathway (UPP) and autophagy-lysosomal pathway. These pathways are carefully regulated by various signalling molecules that ultimately determine protein turnover rates. They are discussed in more detail below.

1.5.2.2 Ubiquitin Proteasome Pathway and Autophagy

Muscle mass in healthy individuals remains constant due to a balance between synthesis and degradation. Specific regulating molecules may be upregulated in response to certain stimuli. Forkhead box O (FoxO) transcription factors are

phosphorylated and inactivated by phosphatidylinositol 3-kinase (PI3K)-Akt/PKB and translocated into the cell nucleus, where they induce the transcription of E3 ubiquitin ligases, MURF-1 and atrogin-1/muscle atrophy F-box (MAFbx), as well as autophagy-related genes LC3 and Bnip3 (53). This large intracellular system regulates muscle mass in response to tumour factors and circulating cytokines. The ubiquitin specific protease 19 is the deubiquitinating enzyme that has been most studied in muscle wasting. It is upregulated in muscle in catabolic conditions and its inactivation leads to protection from muscle loss (54). Ubiquitin specific 19 regulates protein synthesis and degradation, as well as myogenesis, thereby controlling key processes involved in muscle mass maintenance. RNA and protein analysis on muscle biopsies suggests that components of the UPP are associated with weight loss in cancer patients (55) but global gene expression analysis has been unable to replicate those findings which have been widely described in animal models. Inhibition of proteolytic pathways have been investigated in clinical trials. The drug Bortezomib, a proteasome inhibitor, has not shown any effect on muscle wasting and weight loss (56). MG132, a different proteasome inhibitor, improved muscle mass in animal models, possibly due to a different mechanism of action to that observed in human muscle (57). Evidence for upregulation of markers of the UPP in humans is not as robust as those seen in animals, which is perhaps why these drugs have failed in clinical trials.

Autophagy is a homeostatic mechanism involving lysosomal-dependent degradation of cellular products including damaged organelles, proteins and intracellular pathogens. It works in all eukaryotic cells as a quality control mechanism, preventing accumulation of degenerated proteins. Conditions of stress or starvation induce its upregulation to replace damaged cellular products and mobilise energy stores. It regulates muscle mass and muscle-specific deletions of autophagy genes (e.g. Atg7) in mice have been shown to lead to muscle wasting (55). Markers of increased autophagy have been demonstrated in patients with oesophageal, lung, UGI and pancreatic cancers (55). The pathophysiological role of autophagy in cachexia is not fully understood due to difficulties in assessing the autophagic flux in the clinical setting.

1.5.2.3 Myostatin and Activin A

A recent factor that has been the subject of much research is a member of the TGFβ family – Myostatin. Animals and humans who have non-functional copies of the myostatin gene demonstrate dramatic muscle hypertrophy (58). Secretion from skeletal muscle is the main source of Myostatin. It activates Smad2/3 transcription factor complexes through ActRIIB. Enhanced production of myostatin in mice leads to muscle atrophy, whereas genetic inhibition leads to enhanced muscle mass and fibre size (58). The exact mechanisms via which myostatin leads to muscle loss are unclear, but it may be due to its ability to inhibit Akt and TORC1 pathways. These pathways

enhance protein synthesis as well as the ability of myostatin to inhibit myosatellite cell proliferation (58). Other TGFβ family members, such as Activin A, are upregulated in skeletal muscle during cachexia (59). Activin A is increased following activation of the TNFα/TAK-1 signaling pathway. Many cancers induce altered expression of Activin A in muscle (60). Due to the complex interactions within these pathways, the blocking of one target alone may not be sufficient enough to prevent muscle wasting. The ActRIIB receptor is common to both myostatin and the Activins and its blockade in the rodent C26 model of cancer cachexia was effective in reversing muscle wasting and prolonging survival (61).

Attention has been given to therapeutic agents that target myostatin and the ActRIIB pathway in humans. The human derivative of the myostatin neutralising mouse IgG1 monoclonal antibody Landogrozumab (LY2495655) was investigated in a phase 2 trial in palliative pancreatic cancer patients (62). There was a trend towards decreased overall survival in the treatment groups possibly due to differing chemotherapy regimens between groups. Although there was no significant difference in performance status on treatment, patients with <5% weight loss had better performance-related results. This may be indicative of the fact that LY2495655 prevents muscle loss rather than fully reversing the cachectic process, or that patients with end stage disease and refractory cachexia were selectively recruited. These findings are in contrast to a randomised trial involving elderly patients who had fallen, where

LY2495655 treatment increased lean body mass and improved functional measures of muscle power although the results were not statistically significant (63). Initially thought to be more promising was Bimagrumab (BYM338), a human monoclonal anti-ActRII antibody. It prevents the binding of these ligands to their receptor, promoting muscle hypertrophy, and accelerating recovery from muscle wasting conditions in animals. Results from clinical trials have been mixed, with increases in muscle volume seen in patients with inclusion body myositis, sarcopenia and disuse atrophy following fracture (64–66). However, co-primary endpoints of increasing functionality were again not reached.

1.5.2.4 TWEAK and Fn14

Tumour Necrosis Factor-Related weak inducer of Apoptosis (TWEAK) and its related receptor, fibroblast growth factor-inducible 14 (Fn14), are members of the TNF/TNFR superfamily. They are upregulated in tumours and lead to potent skeletal muscle wasting (67). TWEAK causes myotube atrophy through coordinated activation of the UPP, autophagy and caspases. Animal models have shown antibodies to Fn14 can extend lifespan by inhibiting tumour-induced weight loss (67). Fn14 signalling arises from the tumour itself, as tumours in Fn14 and TWEAK-deficient hosts still developed cachexia that was comparable to wild type mice (67). These animal model results suggest Fn14 antibodies may be a promising approach for the future.

Healthy individuals were investigated following resistance training and cardiovascular exercise. Serial muscle biopsies were taken, and TWEAK and Fn14 protein and mRNA levels were found to be elevated in the recovery period, suggesting that they have a role in skeletal muscle metabolism (68).

1.5.2.5 ZIP14

The metal ion transporter ZRT and IRT like protein 14 (ZIP14) is a newly identified critical mediator of cachexia in mouse models of various types of metastatic cancer. It is a member of the SLC39A zinc transporter family. Induced by TNF-α and TGF-β, it is expressed in the liver, and it aids zinc uptake by cells (69). Its muscle-specific deletion reduces muscle wasting in animal models of cancer cachexia. It does this by reducing the expression of MyoD and Mef2c therefore blocking muscle cell differentiation. ZIP14 mediated Zinc accumulation in well-differentiated muscle cells has been shown to lead to the loss of myosin heavy chains. Muscle-specific deletion reduced muscle wasting in animal models of cancer cachexia (70). Drugs that inhibit ZIP14 offer future promise.

1.5.2.6 Mitochondria and exercise

Targeting mitochondrial dysfunction is a therapeutic opportunity to normalise energy metabolism in catabolic conditions. Exercise is an important regulator of mitochondrial function and skeletal muscle metabolism, but compliance with

exercise regimens in patients with advanced cancer is difficult due to chronic fatigue and co-morbidities (71). The benefits of increased exercise include reduced adiposity, increased muscularity, lower chronic inflammation, increased muscle mitochondrial content and function through PGC-1α expression, and improved insulin sensitivity (72). The exercise mimetic 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR), an adenosine monophosphate-activated protein kinase (AMPK) activator, was investigated in C26 mice (73). Activation of AMPK is associated with a pro-atrophic signal in muscle and demonstrates anti-inflammatory effects. AMPK activation in cachexia – an inflammatory driven muscle condition could therefore be useful. AICAR, the AMPK agonist, suppresses IFNγ/TNFα-induced atrophy. IFNγ/TNFα impair mitochondrial oxidative respiration in myotubes and promote a metabolic shift to aerobic glycolysis. AICAR partially restored metabolic function and prevented muscle wasting in mouse models of cancer (73).

Mitochondrial dysfunction can also be due to disruption of fission and fusion processes. The latter is regulated by mitofusin 1 and 2 (MFN-1/2) and optic atrophy protein 1 (OPA1). MFN-1 and 2 have differing functions in GTP tethering and formation of fusion complexes. OPA1 regulates these processes. The loss of mitochondrial fusion has been shown to lead to muscle wasting in MFN1-and 2 knockout mice (74).

1.5.3 Circulating mediators

Many circulating mediators have systemic effects in the pathogenesis of cachexia. Their mechanisms of action, and controversies surrounding the drugs used to target them, are discussed below.

1.5.3.1 Angiotensin II

Patients with advanced cardiac and renal failure often suffer from cachexia. They also display increased levels of angiotensin II. Injection of angiotensin II into rodents has been shown to cause marked weight loss via the angiotensin II type 1 receptor (AT1R), and this loss was reduced in those given Lorsartan (75). Angiotensin II type 2 receptor (AT2R) has also been shown to be increased in mouse skeletal muscle myoblasts during differentiation, suggesting it plays a role in muscle regeneration (76). In humans, reduced weight loss has also been noted in those patients with heart/renal failure and cachexia who took enalapril (77). Causal network analysis techniques (CAN) have identified angiotensin II as an upstream regulator of cachexia, and raised levels have been associated with poorer patient survival (78). Genetic polymorphisms, and single nucleotide polymorphisms (SNPS) coding for angiotensin converting enzyme (ACE) have been identified in patients with cachexia (45). The role of angiotensin II in cancer patients has not yet been investigated.

1.5.3.2 MIC-1/GDF15

Macrophage inhibitory cytokine-1/growth differentiation factor 15 (MIC-1/GDF15) is associated with metabolic disease processes including obesity and anorexia, and has consistently been shown to induce cachexia in animal models (79). The link between MIC-1/GDF15 in humans was first suggested after increased levels were associated with weight loss in prostate cancer patients (80). MIC-1/GDF15 has been shown to act on the hypothalamus and brainstem to cause anorexia, leading to drastic weight loss and cachexia in mice. Ablation of MIC-1/GDF15 in animal models resulted in increased body weight, whereas overexpression led to lower body weight and fat mass (79). The mechanisms by which MIC-1/GDF15 acts on the hypothalamus are unclear. Studies have identified GFRAL, a member of the glial derived neurotrophic factor (GDNF) receptor α family, as the receptor by which MIC-1/GDF15 binds. It is also impacted by GDF11, which is closely related to myostatin and is a more potent ligand for the ActRII/Alk complex than myostatin (81,82). Raised levels of GDF11 cause cachexia in mice, and raise levels of Activin A leading to further muscle wasting. Increased levels of GDF11 cause upregulation of GDF15 and recruitment of SMAD2/3. Inhibition of GDF15 was shown to improve appetite but did not reverse the GDF11 induced muscle wasting. This was reversed by antibodies to the activin type II receptor (ActRIIB) (83). Human trials of anti-MIC-1/GDF15 are awaited.

1.5.3.3 Proteolysis-inducing factor

A much-debated mediator of increased muscle loss in cancer cachexia is the tumour derived proteolysis-inducing factor (PIF). In animal models it has been shown to induce muscle loss via activation of the UPP (84). It was initially isolated from MAC16 rodents and the urine of patients with cancer cachexia. It acts to increase expression of proteasomes via NFkB thus inhibiting protein synthesis (85). Its existence in humans, however, has proved controversial. Several studies have shown a link between PIF and weight loss in patients with cancer. It is specifically expressed by the tumour and has been found in patients urine. The only longitudinal study on the presence of PIF in 36 patients with gastrointestinal cancer showed that those with PIF present in their urine experienced greater weight loss over time. (86) There was concern, however, that the antibody measured in the urine was not specific to PIF (87). The human cachexia-associated protein (HCAP), which is based on the sequence of PIF, has been identified in the cell lines, metastatic tumours and urine of patients with cachexia. It has also been associated with progression of disease and weight loss in patients with prostate cancer (88). Other groups have, however, failed to demonstrate its presence or link it with markers of weight loss, tumour burden or survival in cancer patients (89).

1.5.3.4 Ghrelin

Ghrelin is an orexigenic gut hormone primarily secreted by the endocrine cells of the gastric mucosa. It is a 28 amino acid peptide that is an endogenous

ligand for the growth hormone (GH) secretagogue receptor. Serum levels have been found to be elevated in cachectic cancer patients (90). Ghrelin increases body weight by increasing appetite, GH secretion and prevention of muscle catabolism (91). It has anti-inflammatory actions through its ability to inhibit TNF-α, CRP, IL-1β, and IL-6 in mouse models. Ghrelin is able to initiate its orexigenic effect by stimulation of the hypothalamus and brain stem neurons, and can increase the expression of cannabinoid-1 and MCH-1 receptors on vagal afferents (92). Ghrelin stimulates GH to spare protein stores at the expense of fats therefore demonstrating GH-dependent and independent anti-cachectic effects (93). The drug developed to target it - Anamorelin - is discussed in the final section of this introduction.

1.6 Immune regulation of cancer cachexia

Tumour and host derived factors lead to a chronic inflammatory and impaired immune state. The dysfunction of the immune system is complex and involves multiple mechanisms characterised by a reduction in monocyte, macrophage, dendritic and natural killer (NK) cell function, ultimately leading to susceptibility to infections and poor clinical outcome.

1.6.1 Immune regulation in the host circulation

1.6.1.1 Pro-Cachectic cytokines

1.6.1.1.1 TNF-α

TNF-α [previously known as "cachectin") (94) was initially held responsible for causing most of the metabolic derangements and clinical features seen in cachexia (figure 7). TNF-α is released by many types of cell including activated macrophages, CD4+, neutrophils, mast cells, eosinophils and neurons. In particular, it can be produced by tumour, immune and stromal cells to induce growth and survival advantage in the tumour microenvironment (95). Its expression can ultimately lead to anorexia, muscle and adipose wasting, loss of appetite, increased energy expenditure and insulin resistance in both patients with various types of cancer, and the Colon-26 carcinoma mouse model of cancer cachexia (C26) (96). Many of TNF-α's effects come about through activation of NFkB, which in turn leads to activation of the UPP and

inhibition of myogenesis in vitro (97). It also acts to induce oxidative stress and nitric oxide species (NOS). Experimental evidence suggests TNF-α can induce adipose wasting in white adipose tissue (WAT) through inhibition of lipoprotein lipase (LPL), suppression of transcription and increasing lipolysis as well as stimulating thermogenesis through increased expression of UCP2 and UCP3 in skeletal muscle (98,99).

The role of TNF-α in mediating many of the effects of cancer cachexia was initially supported by evidence that intraperitoneal injection of soluble recombinant human TNF-receptor antagonist was able to improve food intake and thus lead to weight gain in tumour-bearing rats (100). Lewis lung carcinoma (LLC) mice deficient in TNF-α receptor protein type 1 showed a reduction in muscle wasting compared with LLC wild-type mice despite similar levels of TNF-α being detected in the serum (101). Treatment with antioxidants or NOS inhibitors was shown to increase body weight and prevent muscle wasting in mice (102). Despite this, TNF inhibition alone in animals has not been shown to be sufficient to stop or reverse the cachectic process indicating that, although it is involved in the pathogenesis of cancer cachexia, it is not solely responsible (103).

Studies in patients with cancer, however, have not confirmed these effects; in particular, adipocytes taken from cancer patients, showed no decrease in LPL messenger RNA (mRNA) or LPL enzyme activity (104). Some studies have

shown raised TNF-α levels in the serum of patients with pancreatic cancer associated with weight loss, whereas other studies in patients with terminal cancer showed no association between TNF-α and weight loss (105,106). Others have shown that TNF-α correlates with stage of disease or tumour size rather than degree of weight loss (107). These discrepancies between studies may be due to differences in measuring techniques, possible auto or paracrine roles for TNF-α in adipose tissue, or heterogeneity between patients, sexes and tumours.

Although many animal models of cancer cachexia have been shown to synthesise and secrete TNF-α, the actual primary source in humans remains unknown. It is secreted from activated macrophages which have been located in adipocytes in cancer patients. However, subcutaneous adipose tissue biopsies have not shown any changes in inflammatory genes during microarray studies in patients with gastrointestinal cancer (98,108). The presence of innate immune cells in skeletal muscle has not been described as so it is unlikely to be the source. In summary, TNF-α is involved in systemic inflammation but as cachexia is likely to be multifactorial it is difficult to implicate TNF-α as the sole cause. More clinical studies are required to fully identify its effects in patients.

1.6.1.1.1.1 TNFα inhibitors

There are currently many TNF inhibitors in use for the management of other diseases such as rheumatoid arthritis, psoriasis and inflammatory bowel

disease, namely Etanercept, Infliximab and Adalimumab (109). These drugs have revolutionised the treatment of rheumatoid but have also offered insights into the role that TNF-α plays in cachexia. In rheumatoid patients, they attenuate the hepatic acute phase response and importantly improve patient's QoL (109,110). They have also been shown recently to prevent worsening of the disease and restore fat free mass (111). These drugs are now used to treat many thousands of patients and have been shown to be effective at blocking TNF-α but are not effective in treating cancer-induced cachexia. The feature common to all of these diseases is chronic inflammation due to exaggerated production of pro-inflammatory cytokines. The causes of cancer cachexia are complex and do not appear to be dependent upon a single cytokine or inflammatory mediator. Etanercept has showed some promising results in improving fatigue in a small cohort of cancer patients (112). A phase I/II study was conducted on pancreatic cancer patients comparing etanercept and gemcitabine with gemcitabine alone. A small increase in progression free survival was seen and associated with higher plasma IL-10 levels but did not show any significant improvement in 6 month progression free survival compared with gemcitabine alone (112). A placebo-controlled double blind trial was undertaken in 63 patients with incurable malignancy and weight loss of >2.27 kg over 2 months or daily intake of <20 calories/kg body weight. Weight gain was found to be minimal in both arms with comparable survival times. Etanercept was associated with higher neurotoxicity (113). In this trial

therefore it appeared to not be effective in the treatment of cachexia in patients with advanced disease.

Infliximab has been used in a phase II placebo controlled randomised study in patients with stage II-IV pancreatic cancer (114). Patients were given either 3mg/kg or 5mg/kg infliximab and gemcitabine or placebo and gemcitabine. The mean change in lean body mass was +0.4 kg for those on placebo, +0.3kg for those receiving 3mg/kg of infliximab, and +1.7kg for those receiving 5mg/kg of infliximab. No statistically significant differences were seen (114).

Another agent with anti-inflammatory activity is OHR/AVR118, a broad-spectrum peptide-nucleic acid immune modulator that targets both TNF-α and IL-6 (115). A phase II study involving patients with advanced cancer and cachexia showed an improvement in anorexia, dyspepsia, strength, and depression (115).

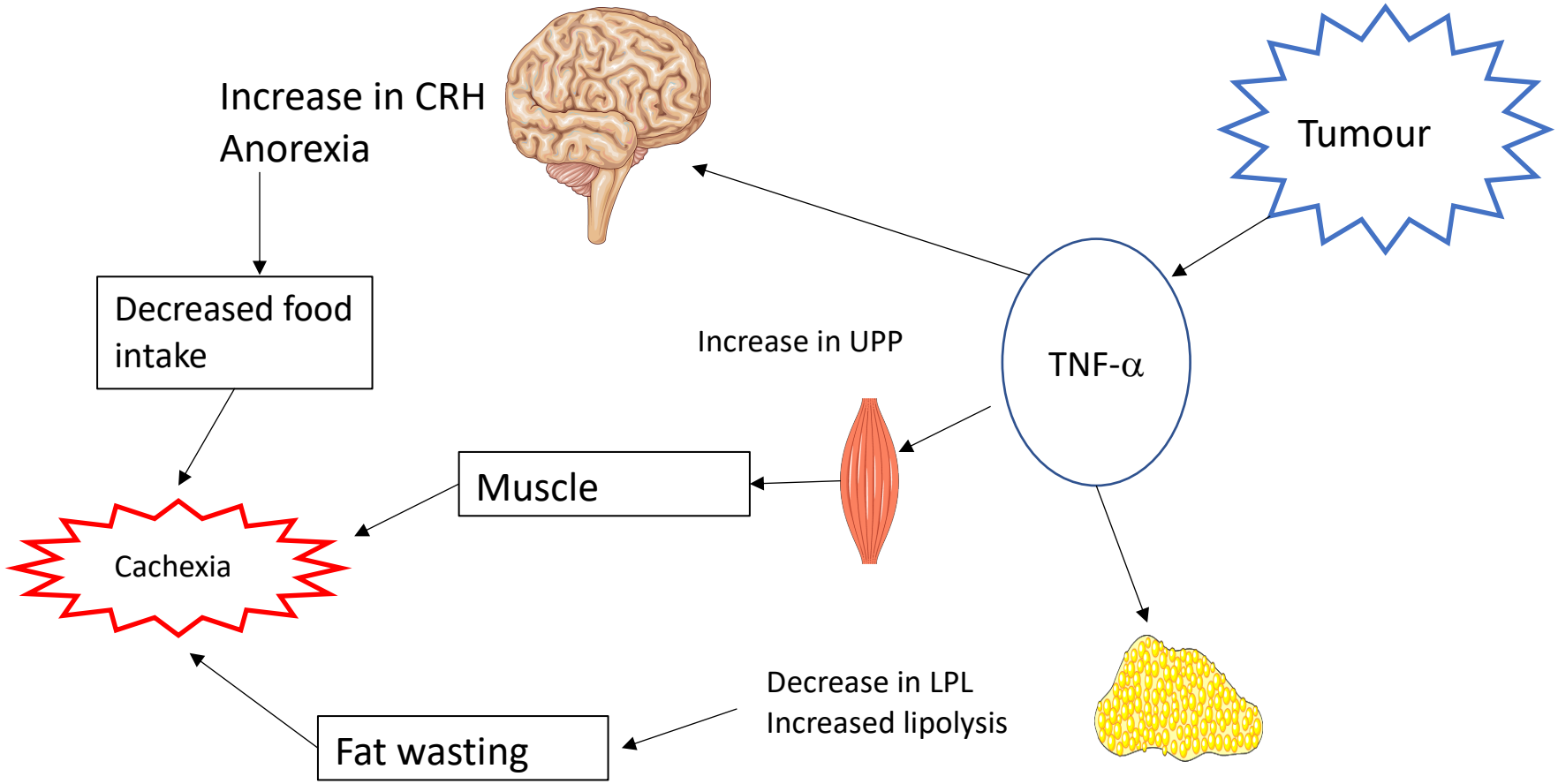


Figure 7 Mechanisms of action of TNFα. TNFα is responsible for a large and varied amount of systemic effects seen in cachexia

1.6.1.1.2 IFNγ

Interferons are multifunctional cytokines that block viral infections and affect cell proliferation and differentiation (116). Interferon gamma (IFNγ) is produced by activated T and natural killer cells, and is arguably the most potent monocyte-macrophage activating factor (117). In the context of cancer, tumour-infiltrating lymphocytes (TILs), which have shown to be of particular importance in tumour immunosurveillance, are the main source of IFNγ (118). There is an overwhelming body of evidence for both beneficial and detrimental roles of IFNγ in a range of diseases, including cancer. However, its role in patients with cachexia is a relatively underexplored area.

Several animal studies have indicated a central role for IFNγ in the pathogenesis of cachexia. Central administration of rat interferon resulted in decreased food intake whereas peripheral administration failed to do so (119). Mice overexpressing IFNγ developed loss of body weight, atrophy of adipose tissue, and reduced appetite, all of which were then reversed by pre-treatment of the mice with anti-IFNγ antibodies (120). Mice with LLC also demonstrated a reduction in weight loss after treatment with anti-IFNγ antibody, significantly reducing fat wasting in particular (121). In rats that had received transplants of MCG 101 sarcoma, anti-IFNγ antibody reduced weight loss but the effect of treatment was short-lived (122). Similarly to TNF-α, IFNγ has been shown to inhibit LPL activity in adipocyte cells in vitro, as well as that of glycerol phosphate dehydrogenase in cultures of rat adipocytes (123).

1.6.1.1.2.1 Anti-IFNγ treatments

Anti IFNγ treatments have been effective in reverting cachexia in the LLC mouse model (120). It has shown no benefit in improving cachexia due to sepsis in humans (124), but there have been no trials undertaken in cancer patients, mainly due to the fact that this type of therapy requires a large number of antibody molecules to completely block the action of IFNγ which at present is very expensive.

1.6.1.1.3 IL-1α

Levels of IL-1 have been shown to be increased in animal models of cachexia. It is thought to cause similar effects to that of TNF-α (125). IL-1 is a pro-inflammatory cytokine produced mainly by macrophages and endothelial cells and is known for being a trigger of the acute phase response, thus playing a role in cancer pathogenesis, as well as shock and autoimmune disorders (126). In a similar way to other cytokines discussed, it is also able to inhibit LPL activity and stimulate lipolysis in cultured adipocytes (127). The ability of IL-1 to induce anorexia is thought to be due to a central effect on appetite suppression involving blockade of neuropeptide Y (NPY) (128). It also increases plasma concentrations of tryptophan and serotonin leading to early satiety and suppression of hunger (129).

Again, there is evidence for the role of IL-1 in animal models of cachexia, but little in humans. IL-1α can induce cachexia and anorexia in rats. IL-1 treated rats showed loss of body weight. Administration of IL-1 receptor antagonist (IL-1ra) to tumour bearing rats however, did not result in any improvement in body weight (130). Following direct tumour injection with IL-1ra, C26 mice demonstrated significantly reduced weight loss (without an effect on tumour burden) compared with mice who had systemic injection. Cultures of C26 cells also demonstrated raised levels of IL-6 after stimulation with IL-1, which were suppressed by monoclonal antibody to IL-6 (131). In tumour samples from patients undergoing surgical resection for upper gastrointestinal malignancy, IL-1β and IL-6 were also significantly overexpressed at both mRNA and protein levels in the cancer specimens compared with control mucosa. Protein levels were seen to correlate with C-Reactive Protein (CRP), indicating that tumour may be the source of IL-1β (132).

1.6.1.1.4 IL-6

IL-6 signals through the membrane bound receptor gp130 found in most tissues in the body (133). IL-6 can target adipose tissue, skeletal muscle, gut, and liver tissue, which can all affect cachectic patient body composition. Once bound to its receptor, it activates JAK tyrosine kinase leading to phosphorylation of tyrosines and the binding of STAT proteins. STAT proteins can translocate to the nucleus and increase the transcription of genes involved in immune function, cell proliferation, differentiation and apoptosis (133).

Several mouse cancer models have clearly demonstrated that blocking IL-6 and associated signalling can attenuate cachexia progression. Deletion of the IL-6 gene in the $APC^{Min/+}$ mouse prevented the development of cachexia (134). IL-6 when secreted by tumour cells can also increase autophagy in myotubes when joined with soluble IL-6 receptor (135). IL-6 trans-signalling through the soluble IL-6R has the potential to amplify IL-6 signalling in the cachectic patient and has been shown to be involved in cross-talk between tumour, muscle and adipose tissue in genetic mouse models of pancreatic cancer cachexia (136). Autophagy and increasing IL-6 levels have been associated with poor prognosis and weight loss in lung cancer patients (135). IL-6 remains a promising therapeutic target in cancer cachexia but a better understanding of its direct and indirect effects, as well as tissue specific actions, are required.

1.6.1.1.5 IL-1/6 inhibitors

The IL-1 pathway has been previously targeted with the recombinant human IL-1 receptor antagonist Anakinra and the neutralising monoclonal anti-IL-1 antibody Canakinumab. Anakinra, as previously discussed, has had success in rheumatoid patients but has yet to be trialled in patients with cancer (109). A more specific IL-1α human monoclonal antibody, MABp1 has also shown promising results. An initial dose escalation and expansion study was designed using MABp1 (137). The first dose escalation part was performed in patients with refractory cancer to assess its safety and tolerability. It identified an optimal intravenous dose which was then used in the following phase II

study of forty-two patients (137). Median plasma IL-6 concentrations decreased from baseline to week 8 (p=0.08). Of those 30 patients who had an assessment of body composition lean mass increased significantly by 1.02 ± 2.24 kg (P = 0.02). It was then compared to megestrol acetate in patients with advanced colorectal cancer and >5% weight loss. Those in the MABp1 treatment arm showed a trend towards increased survival (138). A placebo-controlled, double blind phase III study in 333 patients with advanced colorectal cancer was then undertaken which resulted in increased lean body mass as well as symptom relief (pain, anorexia, fatigue) (139).

IP1510 is a synthetic peptide IL-1 receptor antagonist. Pre-clinical studies suggested it to have low toxicity and be effective for the treatment of cachexia. It was then trialled in advanced gynaecological cancer patients where it was well tolerated, and it significantly improved patient anorexia, depression and physical performance. Weight stabilisation or gain was seen in 17 of the 26 enrolled patients (140). Interpretation of the current data is limited because the study was neither randomised nor controlled. However, further larger trials are to be initiated for cachexia associated with COPD, as well as cancer.

The only current treatment which specifically targets IL-1α is Bermekimab; a human monoclonal IgG1κ antibody. It is cloned from the natural human immune response which neutralises IL-1α. It has been shown to block the

biological activities of IL-1α in vitro by binding to the IL-1R1. It was first studied in patients with a variety of metastatic cancers. It was shown to be tolerable and have minimal adverse events as well as being associated with a 1kg increase in lean body mass (141).

Studies involving IL-6 antibodies have been undertaken in patients with advanced non-small cell lung cancer. The humanised monoclonal IL-6 antibody Clazakizumab (ALD518) has shown beneficial results in increasing haemoglobin levels and preventing loss of lean body mass. Fatigue scores were also improved compared with controls (142). There are, however, no phase III trials underway.

1.6.2 Cells

1.6.2.1 Myeloid derived suppressor cells

Many studies have now suggested that tumour infiltrating immune cells (those which are mainly of myeloid origin) are able to differentiate into cells which then promote tumour growth and metastasis through their ability to induce systemic inflammation (143). Tumours can grow through myelopoiesis and the successful evasion of tumour cells from both the innate and adaptive immune systems (144). However, the progression of cancers appears dependent on tumour-associated myeloid cells through their ability to promote angiogenesis and tissue remodelling (145). This apparent immunosuppression has been

linked to the development of cachexia in very few studies, despite tumour-induced immunosuppression being well documented in the literature (146).

Myeloid derived suppressor cells (MDSC) may play a role in tumour related immunosuppression. Tumour-induced amplification of the myeloid compartment leads to the expansion of MDSC. MDSC are immature myeloid cells in various stages of differentiation but are not fully differentiated neutrophils, monocytes/macrophages or dendritic cells. They are found in the bone marrow, spleen, lymph nodes and tumours (143). Their mechanisms of action are not fully understood but they are thought to be immunosuppressive and to play a role in the over production of cytokines and inflammatory mediators which may contribute to cachexia.

MDSC expansion in 4T1 breast carcinoma-bearing mice is associated with the induction of the hepatic acute phase protein response and altered fat metabolism (146). This response is also seen in the C26 and LLC mouse models. The cachectic response is not seen, however, in 66C4 subclone of 4TI mice in which MDSC expansion does not occur (146). Defects in myeloid cell-mediated inflammation has also been shown to result in reduced expression of pro-inflammatory cytokines in the serum of mice with hepato-cellular carcinoma (147). Interestingly, this led to enhanced loss of adipose tissue and decreased macrophage number in visceral adipose tissue, suggesting a possible local role for macrophages in the regulation of cancer-

induced fat loss (147). These findings imply that myeloid cell-mediated inflammation confers a beneficial function in these rodents, and may provide a potential explanation for the failure of several anti-inflammatory drugs in treating cachexia. Although a direct link between the development of cachexia and MDSC's has not been proven, the above studies have suggested that the development of cancer cachexia is partly explained by the expansion of immature myeloid populations associated with the tumour.

1.6.3 Immune system biomarkers

The Neutrophil:Lymphocyte ratio is a prognostic indicator in cancer. Neutrophils increase the inflammatory reaction to pathogens but also interact with cancer cells to produce cytokines and effector molecules e.g. VEGF that are able to stimulate angiogenesis and promote tumour growth (148). Activated neutrophils can move from the circulation to the tumour site to release reactive oxygen species which in turn can lead to further DNA damage. They also have anti-tumour roles through antibody-mediated cytotoxicity of tumour cells (149).

CRP is an acute phase non-specific inflammatory marker that can be elevated in response to infection, surgery or malignancy. It is produced by the liver in response to increased levels of IL-6 released by activated macrophages as well as IL-1 and TNF-α (150). The Glasgow Prognostic Score utilises raised CRP and hypoalbuminaema to predict those patients with cancer cachexia

who have a poor outcome. It has been examined in more than 60,000 patients and has been shown to have independent prognostic value (151).

1.6.4 The role of immune cells in the tumour mass

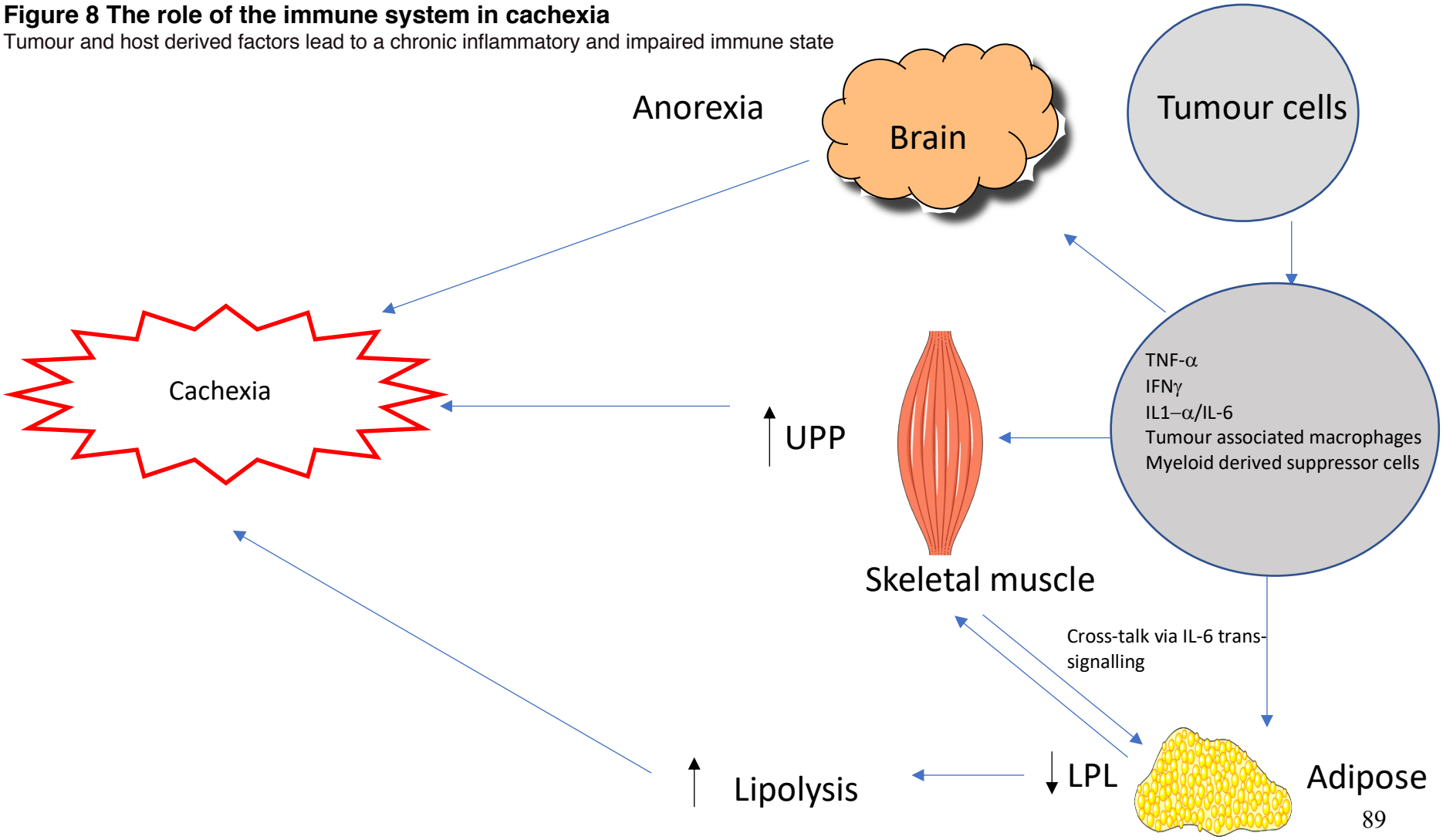
1.6.4.1 Tumour associated macrophages

Tumour associated macrophages (TAMs) increase tumour progression and metastasis and suppress anti-tumour immune functions (152). Monocytes from blood infiltrate the tumour and are primed by the tumour microenvironment to exert these effects. The immune cells within the tumour's microenvironment consist of myeloid-derived suppressor cells, NK cells, dendritic cells, T cells and macrophages (153). It is this infiltrate that contributes to tumour growth and the release of cytokines that promote the pro-cachectic environment. TAMs are recruited via cytokines and chemokines and suppress the activity of cytotoxic T-lymphocytes via programmed cell death 1 ligand 1 (PD-L1) or B7-H4 and other receptors (154). Activated macrophages also secrete cytokines leading to the activation of several complex cascades, thereby increasing inflammatory status and worsening cachexia (155). The chemokine monocyte chemotactic protein-1 (MCP-1) is possibly responsible for the migration of monocytes to adipose tissue in chronic inflammation (156). The mechanisms by which macrophages modulate adipocyte function in cachexia are still unclear.

1.6.4.2 Tumour infiltrating lymphocytes

Tumour infiltrating lymphocytes (TIL) are often found in tumours and are thought to reflect an immune response against the tumour. Many studies report a survival benefit associated with the presence of TIL suggesting they may delay tumour progression. CD3+ and CD8+ TILs in particular have been identified as having a positive effect on prognosis (157).

Figure 8 The role of the immune system in cachexia
Tumour and host derived factors lead to a chronic inflammatory and impaired immune state



1.6.5 Difficulties in immune regulation of cancer cachexia

The cytokines linked to cachexia development in cancer patients appear to be dependent not only on the type of tumour but also on the burden of disease present, and patient specific factors such as age, sex and genotype. It is still not fully understood why one patient with the same tumour type as another may become cachectic whereas another may not. Genetic variation in immunity suggests one possible reason for this. Single nucleotide polymorphisms in the IL-1, IL-6 and IL-10 genes have been associated with cachexia in gastrointestinal cancers (158). The 1082G allele in the IL-10 promoter was validated in an independent cohort. This was shown to be increased in Myc/mTOR-driven mouse models of cachexia as well as cachectic colorectal cancer patients (158). The C allele of the rs6136 polymorphism in the P-selectin gene has also been associated with weight loss and low CT muscularity in a large group of cancer patients (45). These results suggest a role for the immune system in the complex presentation of cachexia.

It is also important to consider the complexities involved in the use of anti-cytokine drugs in patients with cachexia. Given that immunosuppression is a key component of cancer pathogenesis and therefore potentially cachexia, these treatments may carry theoretical risks of carcinogenesis or disease progression. Common side effects often include reduction in neutrophil counts

thus making these already sick patients more susceptible to infections and further risk of different cancers.

1.7 Tissue cross-talk

The importance of tissue cross-talk in cachexia pathophysiology is gaining more attention. In particular, fat-muscle cross-talk and circulating microRNA mediators of systemic cross-talk are developing research interests.

1.7.1 Fat-Muscle cross-talk

Animal models have indicated a role for fat-muscle crosstalk in cachexia. In particular, inhibition of lipolysis through genetic ablation of adipose triglyceride lipase (Atgl) or hormone sensitive lipase (HSL) reduced muscle wasting (figure 9) (159). The mediators of cross-talk include myokines, adipokines or free fatty acids. A previous study has described worsening myosteatosis in association with increasing degrees of cachexia. (160,161) The infiltration of adipose tissue into muscle during the cachectic process may therefore be another contributor to wasting. This supports the loss of visceral fat driven by lipolytic mechanisms as an early event in cancer cachexia with a potential effect of increasing fat deposits in skeletal muscle. Inhibition of fatty acid oxidation with Etoxomir (an inhibitor of carnitine palmitoyltransferase-1) has been shown to stop muscle wasting in animal models (162,163). Parathyroid hormone is thought to cause adipose tissue browning and increased energy production

via uncoupling protein 1 (UCP1) through activation of protein kinase A (PKA). It is also contributes to muscle wasting through activation of the UPP (164). Treatment with an antibody neutralising the parathyroid hormone related protein (PTHR) prevented muscle wasting as well as adipose tissue browning and loss in Lewis Lung Carcinoma-bearing mice (165).

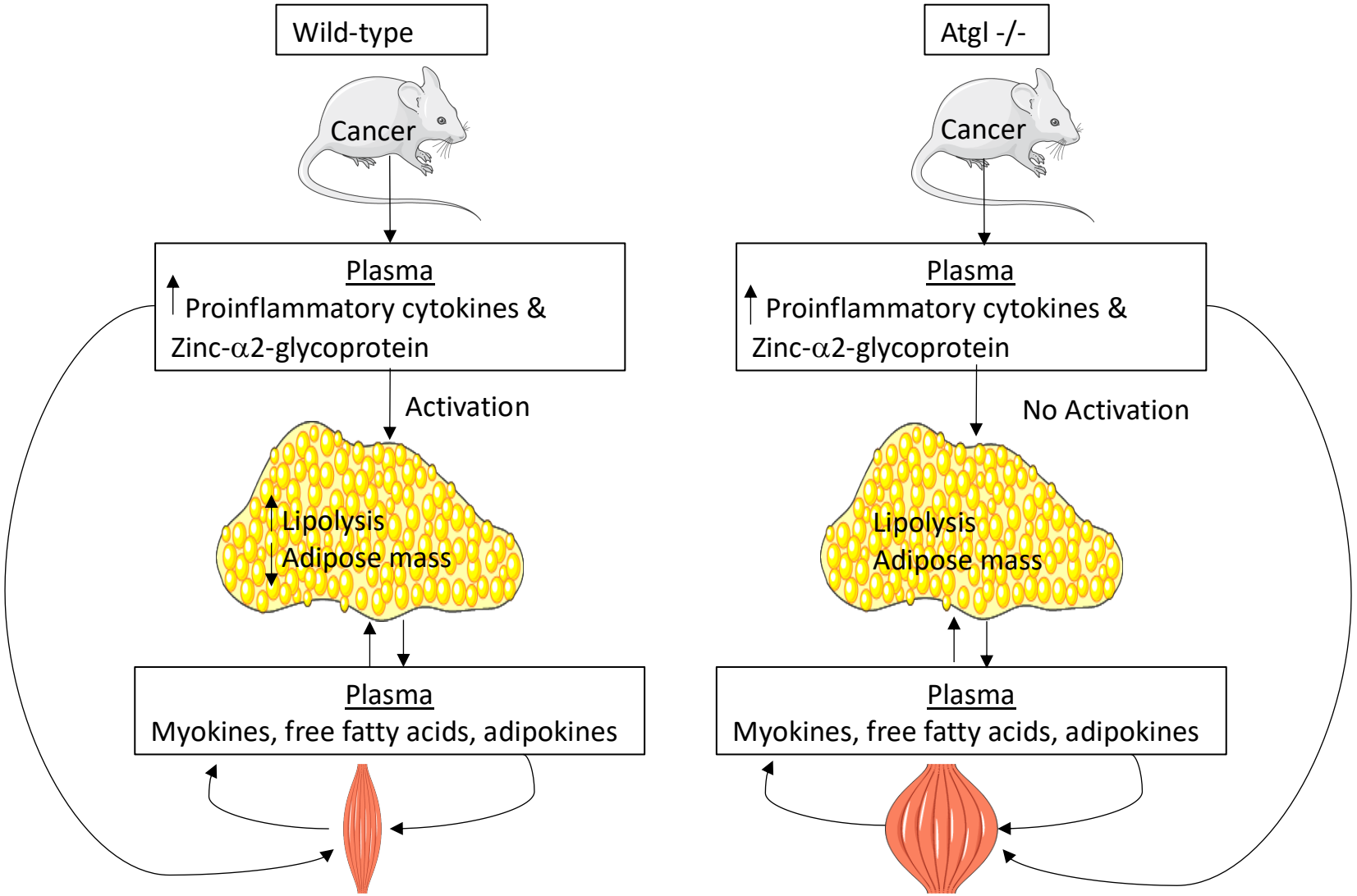


Figure 9 Fat-muscle cross-talk in cancer cachexia adapted from 157 Inhibition of lipolysis reduces muscle wasting

1.7.2 Involvement of other tissues

Many other organs or tissues are involved in cancer cachexia, and the cross-talk between such organs may be driven by systemic inflammation. The liver is a major responder to systemic inflammation by increasing the production of acute phase proteins. Effects on cardiac function lead to wasting and decreased innervation, possibly causing death (166). The brain promotes changes in neuropeptides that regulate appetite, and activation of the hypothalamic pituitary axis leads to the release of glucocorticoids, which act to promote muscle wasting. Loss of muscle is associated with increased osteoclast function and therefore increased bone resorption, worsening function and QoL (167).

1.7.3 Microvesicles and MicroRNAs

The study of exosomal micro RNAs (miRNAs) and muscle-specific miRNAs (myomiRs) is a promising future field of research for cancer cachexia. Both are thought to play a role in the transduction of inflammatory signals and the activation of catabolic status in muscles (168). miRNAs are embedded in microvesicles, which are small membrane-derived particles that are potentially able to communicate with other cells in the body by fusing with their plasma membrane, thus allowing the delivery of various molecules including miRNAs, mRNA and proteins (169). High levels of tumour-released extracellular Hsp 70 and 90, in particular, have been detected in the serum of cachectic mice (168).

Exosomal-transported miRNAs are of particular interest as they are involved in the development and continuation of inflammation in cancer cachexia. Exosomes are nanovesicles which promote tissue cross-talk in an autocrine, paracrine and endocrine manner. MiRNAs make this communication more efficient. They are non-coding RNA molecules that regulate gene expression through the degradation of mRNA and have already been shown to modulate inflammatory pathways, induce metastasis, mediate cancer aggressiveness, and participate in the regulation of protein synthesis and degradation in skeletal muscle.

Changes in the profile of miRNAs may indicate the presence or progression of muscular disease. Several studies have shown an increase in miR-21 in patients with a variety of cancers (colorectal, gastric, prostate and liver) (170,171). The upregulation of miR-21 is thought to promote muscle atrophy by binding to the transcription factor YY1, as well as the translational initiation factor eIF4E3. In binding to these, miRNAs are able to regulate muscle mass wasting and interfere with myogenesis (168). By analysing rectus muscle biopsies, eight other miRNAs that are upregulated in cancer cachexia patients were demonstrated. These include: hsa-let-7d-3p; hsa-miR-345-5p; hsa-miR-423-5p; hsa-miR-532-5p; hsa-miR-1296-5p; hsa-miR-3184-3p; hsa-miR-423-3p; and hsa-miR-199a-3p. These miRNAs affect muscle mass in a variety of different ways by controlling appetite, interacting with transferrin and insulin-

like growth receptors, as well as down regulating genes involved in lipid biosynthesis, leptin and energy balance (172).

Taken together, these findings demonstrate the role of miRNAs and myomiRs in the modulation of atrophic pathways in cancer cachexia. As cachexia is a syndrome of systemic effects, in which there is thought to be a large amount of tissue cross-talk, these particles are likely to play an important role. Improved knowledge about their characterisation and functions, particularly of exosomal miRNAs, would aid in the quest for a cachexia-related biomarker, or aid in the development of a drug which could diminish the release of tumour-induced inflammatory factors.

A summary of some of the novel mediators and mechanisms discussed is shown in figure 10.

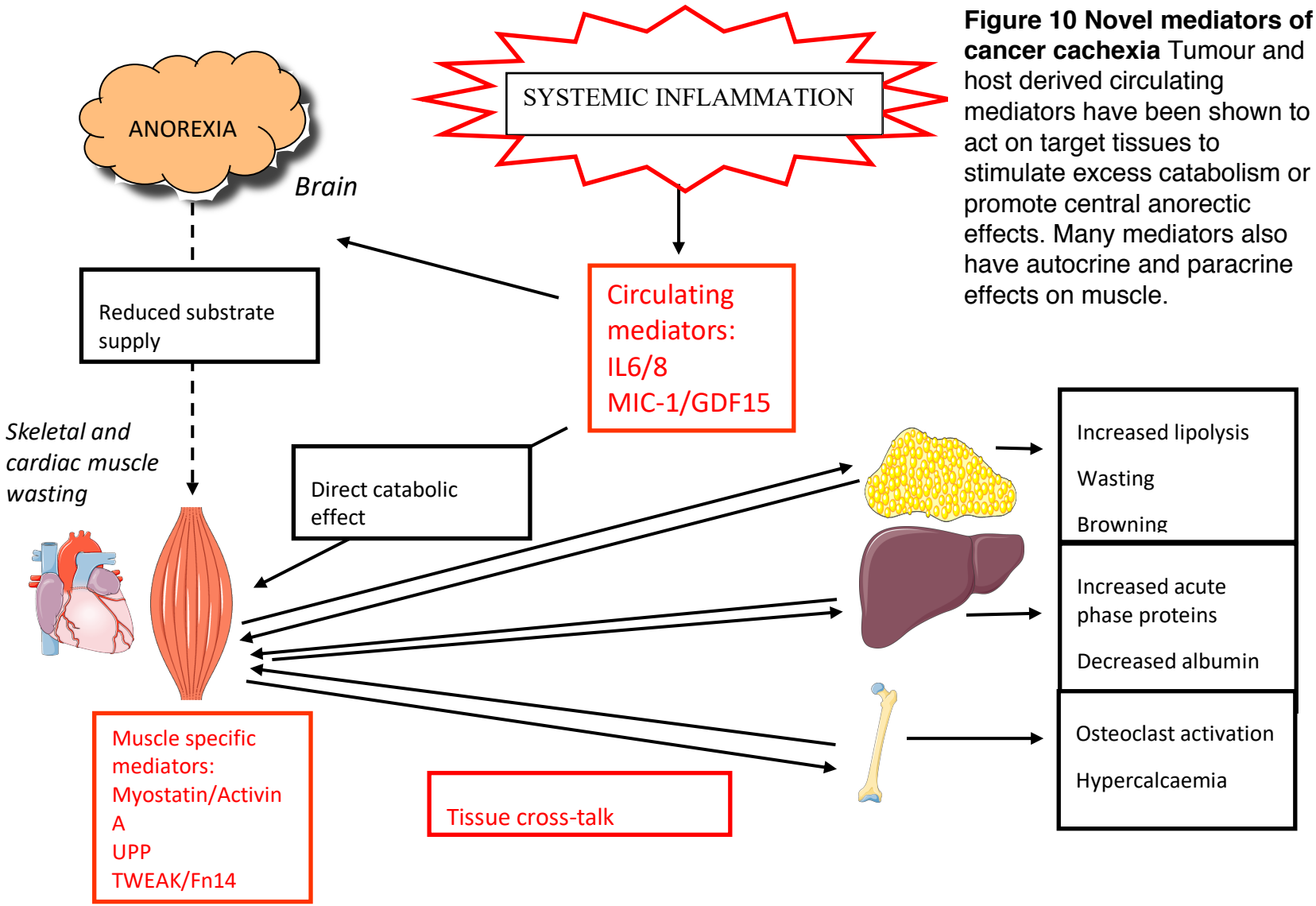


Figure 10 Novel mediators of cancer cachexia Tumour and host derived circulating mediators have been shown to act on target tissues to stimulate excess catabolism or promote central anorectic effects. Many mediators also have autocrine and paracrine effects on muscle.

1.8 Cross-sectional imaging

The next section will detail current imaging methods used to diagnose or screen for the pathophysiological changes described above.

Within both the international consensus definition of cachexia and the European Working Group on Sarcopenia in Older People (EWGSOP) statement on age-related sarcopenia are defined cut-offs below which the patient is considered to have low muscle mass (28,173). DXA was the first imaging modality used to investigate this (174). It correlated well with measures of total body potassium, a marker of body cell mass, therefore validating its use (175). Imaging techniques and body composition analysis have since developed. The benefits and limitations of the most popular techniques currently in use are discussed below.

1.8.1 Computed Tomography

CT is often used over time to monitor cancer progression or response to treatment and can therefore be taken advantage of to investigate body composition. CT can differentiate between skeletal muscle, adipose tissue, bones and organs based on tissue specific attenuation values using newly developed software programmes, including SliceOmatic and ABACS, which has been used in this thesis (TomoVision, Canada). This programme is designed to be used at the third lumbar vertebra, muscularity at which has

been shown to correlate with total body skeletal muscle volume (32). This technique gives a measurement of cross-sectional area (CSA), which can then be indexed against height to give the skeletal muscle index (SMI) in cm^2/m^2. This was developed in a cohort of 1473 patients with gastrointestinal and respiratory cancers (27). The cut points developed were based on those patients who had reduced survival time. They were then analysed further to produce cut points which accounted for BMI and patient sex. It is important to note, however, that these cut points do not take into account any assessment of muscle function and therefore questions have been raised about their ability to diagnose true sarcopenia (176,177). CT also does not take into account the fact that muscle wasting is a dynamic process and it therefore provides an assessment of muscle mass at a defined, static time point. CT has, however, been widely validated in a range of studies across oncological and surgical fields as a method of body composition analysis and continues to be used to highlight that low SMI remains an adverse prognostic indicator.

1.8.2 Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) is a highly accurate method to measure body composition and is comparable to CT without exposing patients to ionizing radiation (173,178). It is often used in other non-cancer patient groups to monitor effect of muscular injuries or disease. Measuring body composition from MRI is, however, more complex. The MRI scan often uses the quadriceps muscles as a marker of total body musculature but also allows the entire

volume of muscle to be scanned rather than estimating it from SMI. Each scan takes a much longer period of time and interpretation is very labour intensive (179).

1.8.3 Dual X-Ray absorptiometry

The most common method for evaluating body composition in cancer cachexia clinical trials has been DXA. It is used to measure total appendicular lean tissue, including the extremities, and is therefore assumed to be representative of muscle mass (180). Its use in assessing body composition in oncological clinical practice is restricted as it has several limitations in this setting, including the burden of an additional scan and associated costs. Due to this the international consensus definition recommends CT or MRI as first choice imaging over DXA in cancer patients (28).

1.8.4 Bioelectrical impedance analysis

Bioelectrical impedance analysis (BIA) can be a useful tool for assessing skeletal muscle mass and is accepted as an option for doing so by the EWGSP, Asian Working Group for Sarcopenia and the International Consensus for Cancer Cachexia (28,181). It differs from other body composition tools in that it does not measure a specific body component. It relies on calibration equations developed from other reference methods e.g. CT or DXA. These equations also assume body shape, the relationship between trunk and leg lengths, and the level of hydration and fat fraction (182).

Earlier BIA systems used fat free mass (FFM) prediction equations developed using underwater weighing or total body water. However, new equations have been developed to identify body components other than FFM and percentage fat. Phase angle is a more up to date part of the BIA test which is used as a marker of cell membrane integrity with lower scores being related to cell apoptosis. Cancer reduces intracellular water through cachexia such that the total body water derived from these equations for normal populations are likely to be inaccurate (183).

1.8.5 Contrast Enhanced Ultrasound Scanning

Other imaging techniques are emerging which have been applied to explore the effect of cachexia on skeletal muscle morphology, metabolism and micro-circulation namely Magnetic Resonance Spectroscopy (MRS) and contrast enhanced ultrasound (CEUS) (184). CEUS is able to quantify the skeletal muscle mircro-circulation by analysing the replenishment kinetics after destruction of intravenously injected microbubbles by ultrasonographic impulses (185). It is sensitive enough to allow detection of low skeletal muscle perfusion (capillary blood flow at rest) and also allows for measurement of increased blood flow after exercise or during pathology e.g. inflammatory myopathies. MRS allows for non-invasive analysis of the chemical composition of tissues (185). It is able to quantify concentrations of several metabolites in particular intramyocellular lipids and high energy phosphates which are encountered in diseases leading to muscle degeneration (184). A previous

study investigating these imaging techniques has been undertaken in cachectic patients and healthy volunteers. Lower muscle mass in the cachectic group was confirmed by MRI. Capillary density as determined by histology, microcirculation measured in vivo by CEUS, and concentrations of metabolites analysed in vivo by MRS were the same in both groups. Cancer cachexia was therefore associated with a loss of muscle volume but not of functionality. This is indicative of sufficient oxidative phosphorylation potential and oxygen availability in muscles of patients with cancer cachexia, suggesting therefore that cachectic muscles should respond to exercise programmes since they are apparently not damaged irreversibly (184).

1.9 Clinical consequences and management of cachexia

The relative clinical implications of the pathophysiology described above will now be discussed in more detail.

Cachexia must be considered as a systemic syndrome. Many of the symptoms experienced by patients with cachexia may be attributed to muscle wasting with decreased muscle mass leading to fatigue and the inability to perform simple tasks (186). Patients with cachexia often report altered body image, which has a negative impact on their emotional well-being, as well as family, work and social lives (187) (figure 11). These patients often also suffer from anorexia and fatigue which compounds physical activity problems (186). In a study measuring physical activity levels in patients with cachexia using an

activity monitor, those with cachexia were shown to have a 40% reduction in their levels of physical activity (188).

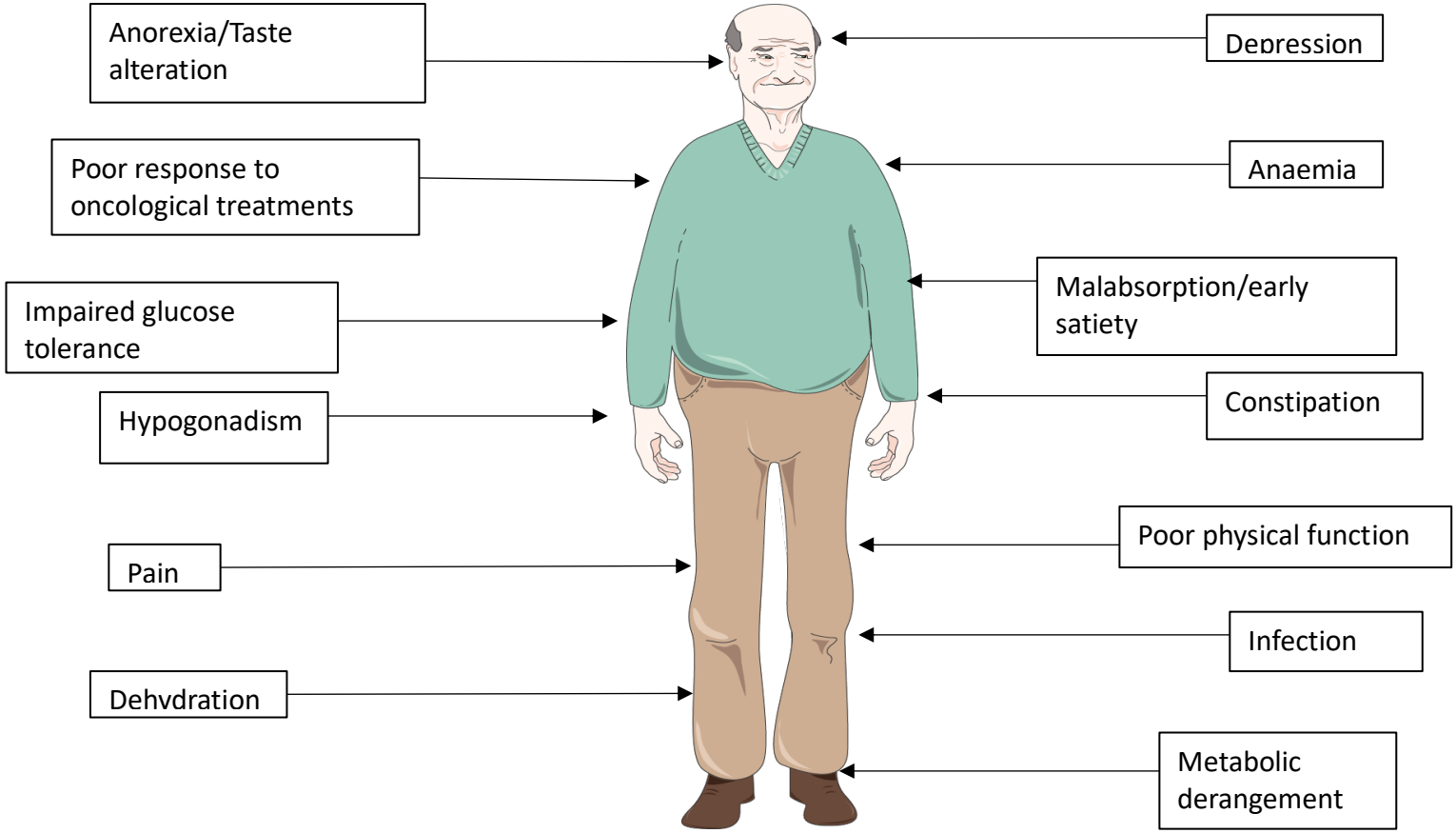


Figure 11 Symptoms associated with cachexia The effects of cachexia are multiple, ranging from severe metabolic derangements to psychological distress, each of which contributes to poorer outcomes after surgical and oncological treatments, and worsened quality of life.

1.9.1 Impact of low skeletal muscle mass on chemotherapy toxicity and survival

There are many CT-based body composition studies in existence investigating the association between low skeletal muscle mass and survival, with most showing reduced survival and poor outcomes. A recent systematic review assessed 53 articles on CT based measures of 9138 patients. Most studies were aimed at assessing the association between survival rates and CT defined body composition (189). Low muscle mass at baseline was a strong predictor of survival independent of age, sex, disease site and performance status. Although this was noted in all patients, those who were obese and had low muscle mass appeared to have the worst survival rates (189).

These patients with low muscle mass on CT also have an increased risk of toxicity to chemotherapy and a reduced time to tumour progression. Chemotherapy drugs are measured on the basis of body surface area which does not account for muscle and fat loss or water retention. As a result the distribution of cancer drugs is impacted and may therefore decrease their effectiveness or increase levels of toxicity. Increased toxicity due to changes in body composition has been shown in patients with breast, gastric and colon cancers (190–192). Patients with metastatic breast cancer had a reduced time to tumour progression for capceitabine and 5-fluorouracil based chemotherapy (191). In patients who had oesophagogastric cancer low muscle mass was found to be present in 57% of patients upon diagnosis. Following neoadjuvant

chemotherapy the patients demonstrated a further loss in FFM and the proportion of patients with low muscle mass increased (193).

Muscle wasting over time also seems to be important. In a study including patients with advanced non-small cell lung cancer patients who received carboplatin-based therapy, skeletal muscle wasting over time but not sarcopenia at baseline, independently predicted survival (194). This may have been due to chemotherapy toxicity. Skeletal muscle mass therefore may be a better predictor of chemotherapy toxicity than body mass and as a result changes in body composition following chemotherapy will be investigated in more detail in chapter 4.

1.10 Complexities of body composition analysis

1.10.1 The relationship between cachexia, sarcopenia and malnutrition

Several conditions present with unintentional weight loss (UWL) namely cachexia, sarcopenia and malnutrition (figure 12). The term sarcopenia relates to loss of muscle mass with aging. Sarcopenia has been defined as “appendicular skeletal muscle mass divided by body height squared in meters (muscle mass index)” two standard deviations or more below reference values from young, healthy individuals measured with DXA (174). Skeletal muscle mass assessed by radiological methods also relates to the term “sarcopenia”. This can be used to describe low muscle mass on CT scan but requires a

reduction in muscle function, as well as mass, to meet the EWGSOP definition (173,195). A number of other definitions of sarcopenia can be found in the literature but currently no term exists to define the radiological existence of low muscle mass; “myopenia” has been previously suggested (196). The theory that decline in muscle function is explained by a decline in muscle mass has also yet to be fully proven.

Cachexia should not be confused with malnutrition; there is a distinct difference. Malnutrition is nutritional depletion following starvation that promptly responds to nutritional supplementation, and replacement. Protective mechanisms are triggered within the body that minimise energy needs by reducing energy expenditure and preserving muscle mass at the expense of fat mass. Cachexia causes these protective pathways to not operate, which accelerates nutritional depletion and progressive loss of muscle and fat. Cachexia is also minimally responsive to nutritional supplementation particularly in the advanced stages of disease (197).

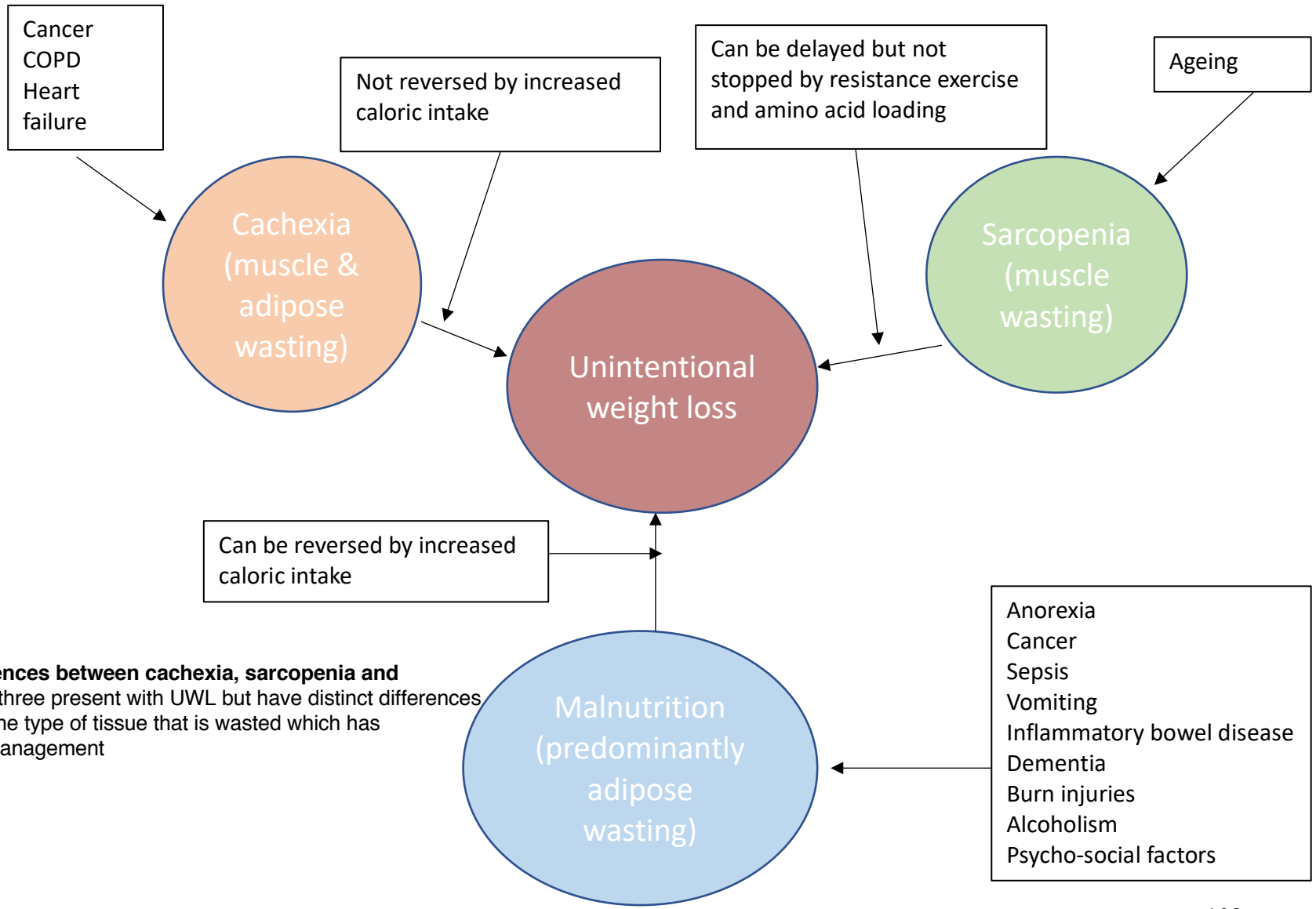


Figure 12 Differences between cachexia, sarcopenia and Malnutrition. All three present with UWL but have distinct differences in aetiology and the type of tissue that is wasted which has implications for management

1.10.2 Sexual dimorphism

There has been considerably more interest of late in the fact that the response to muscle loss and function in patients may be sexually dimorphic. Previously, in gastrointestinal cancer patients, it has been shown that there is variability in lower limb muscle function, mechanical quality, and mass according to both the degree of weight loss and patient sex (198). While lower limb muscle mass, strength and power were shown to decline in male cancer patients, females appeared to experience attenuated loss of muscle mass and power only. Mechanical quality was reduced in both male and female cancer patients, but only in females did it decline progressively with cachexia (198). These changes lead to a significant reduction in patient's QoL. One proposed mechanism to account for sexual dimorphism may be to do with sex hormone production. It has been demonstrated that hypogonadism (low total and/or free testosterone levels) is prevalent and is associated with poorer survival in male cancer patients (199). It is also well established that low testosterone levels impact on muscle mass and function. Testosterone concentrations are known to decline with age and disease (200). It has a role in the repair of damaged muscle by activating myoblasts and increasing satellite cells, therefore leading to increased protein synthesis (201). It has anti-inflammatory properties by stimulating the release of IL-10 (202) and inhibiting the release of TNF-α, IL-1β, and IL-6 (203,204). Lowered concentrations of testosterone are also associated with raised levels of Leptin, which has anorexic and lipolytic effects (174). Furthermore, testosterone replacement has been shown to prevent loss

of muscle in ageing males (205). These findings are consistent with recent literature suggesting sexual dimorphism may impact on the effects of systemic disease such as cirrhosis and must be considered when treating cachexia in clinical practice (206).

A molecular study investigating dimorphism has recently been undertaken. (207). Molecular data were obtained from 41 K Agilent microarray analysis of rectus abdominus muscle and Liquid Chromatography Mass Spectrometry (LC-MS) analysis of urine in patients with cancer. This study was able to identify altered pathways in men and women. Increasing muscularity was associated with JAK/STAT signalling pathways in men and growth hormone (GH) signalling pathways in women and was negatively associated with mismatch deoxyribose nucleic acid (DNA) repair in men and with oestrogen receptor (ER) mediated phagocytosis in women. The urinary proteome and muscle transcriptome suggested altered carbohydrate metabolism in women but not in men. Inflammation, however, was associated with low muscle mass independent of sex (207). The effect of gender has also been investigated in a study of 368 patients with a variety of advanced cancers (pancreas, cholangiocarcinoma, lung and colorectal). The authors concluded that male pancreatic cancer patients lost more muscle and fat at an accelerated rate compared with females (208). These data suggests that future interventions and management of cancer cachexia would be best tackled with a sex-specific approach.

1.10.3 Adipose tissue and cachexia

1.10.3.1 Obesity

Individual patients do not have the same body habitus at diagnosis. Demographics have changed since the original cachexia definition was developed with rates of obesity increasing worldwide (209). Obesity is defined as "abnormal or extensive fat accumulation that negatively affects health." According to the World Health Organisation (WHO) it is defined as a "BMI ≥30 kg/m^2 and waist circumference as >102 cm for men and >88cm for women" (210). Obesity is a well-known risk factor for many types of cancer (211) and is usually considered to have a disadvantage on survival (212), however, it has been shown to confer a survival advantage in patients with weight loss associated with heart and renal failure due to their larger energy reserve (213). Diagnostic criteria for cachexia therefore needs to include additional nutritional information beyond weight loss.

1.10.3.2 Sarcopenic obesity

Older obese persons with decreased muscle mass or strength are at special risk of adverse outcomes. Excess energy intake, low levels of physical activity, low grade inflammation, insulin resistance, and changes in hormones may lead to the development of sarcopenic obesity (214).

Sarcopenic obesity was also first defined by Baumgartner (215). It has since been further defined using anthropometrics, BIA and strength which poses its own problems given there are no accepted criteria for low muscle strength (216). Similarly to prevalence rates in cachexia, rates of sarcopenic obesity vary depending upon definitions used, ranging from 4 to 12% (215–217) (table 2). Many longitudinal studies have shown that fat mass increases with age, with muscle declining after age 30 and being more rapidly lost in the elderly (218–220). These important differences in body composition parameters between age groups will be investigated in chapter 4.

Many patients with pancreatic cancer will be over-weight rather than underweight which makes applying conventional risk stratification measures difficult. In a study of 2115 patients with cancers of the respiratory and gastrointestinal tract sarcopenic obesity was associated with poorer functional status compared with obese patients who did not have sarcopenia and independently predicted survival. Estimated fat free mass (FFM) showed a poor association with body surface area which as discussed above impacts on chemotherapy distribution (221). Sarcopenic obesity therefore is an independent adverse prognostic indicator that should be considered for stratification of patients entering clinical trials, systemic therapy, or supportive care programs.

Study	Definition of sarcopenic obesity	N	Mean age (SD)	Prevalence
New Mexico Aging Process Study (215)	- Sarcopenia: skeletal muscle mass -2 SD below mean of young population or < 7.26 kg/m^2 in men and < 5.45 kg/m^2 in women. - Obesity: percentage body fat greater than median or > 27% in men and 38% in women.	831	60 and over	M: 4.4% F: 3.0%
Nhanes III (216)	- Sarcopenia: two lower quintiles of muscle mass (<9.12 kg/m^2 in men and <6.53 kg/m^2 in women) - Obesity: two highest quintiles of fat mass (>37.16% in men and > 40.01% in women)	M:1391 F:1591	M:76.3 (1.7) F: 77.3 (2.2)	M:9.6% F:7.4%
Zoico et al (217)	- Sarcopenia: two lower quintiles of muscle mass (<5.7 kg/m^2) - Obesity: two highest quintiles of fat mass (>42.9%)	F:167	71.7(2.4)	F:12.4%

Table 2 Prevalence of sarcopenic obesity by various definitions. There is marked variation in prevalence rates dependent on the definition used.

1.10.3.3 Effect of changes in adipose tissue on survival

It is unclear whether it is adipose or muscle wasting or a combination of the two which has the most negative impact on survival. A study based on pooled recent data (222) (n=8160) evaluated the prognostic significance of weight loss in patients who initially had low, intermediate or high BMI. The authors showed that the patients with a lower starting BMI had a worse prognosis, with BMI and weight loss being independent predictors of survival regardless of cancer site and stage. They were therefore able to develop a cancer weight loss grading system that incorporated BMI and weight loss in order to predict the risk of reduced survival as shown in figure 13.

A further study investigated the independent prognostic significance of adipose tissue in predicting mortality in cancer patients (223). Total adipose index (TATI), visceral adipose index (VATI) and subcutaneous adipose index (SATI) were estimated for 1473 gastrointestinal and respiratory cancer patients. Low SATI was independently associated with increased mortality and shorter survival. In the presence of sarcopenia, however, the longest survival was observed in patients with high subcutaneous adiposity (223). Other studies have suggested that those patients who lose fat mass only have as poor a survival as those patients who lose muscle and fat mass (224). The relevance of fat wasting in cachexia does however, remain a relatively unanswered question and the investigation of this will therefore form a key component of this thesis.

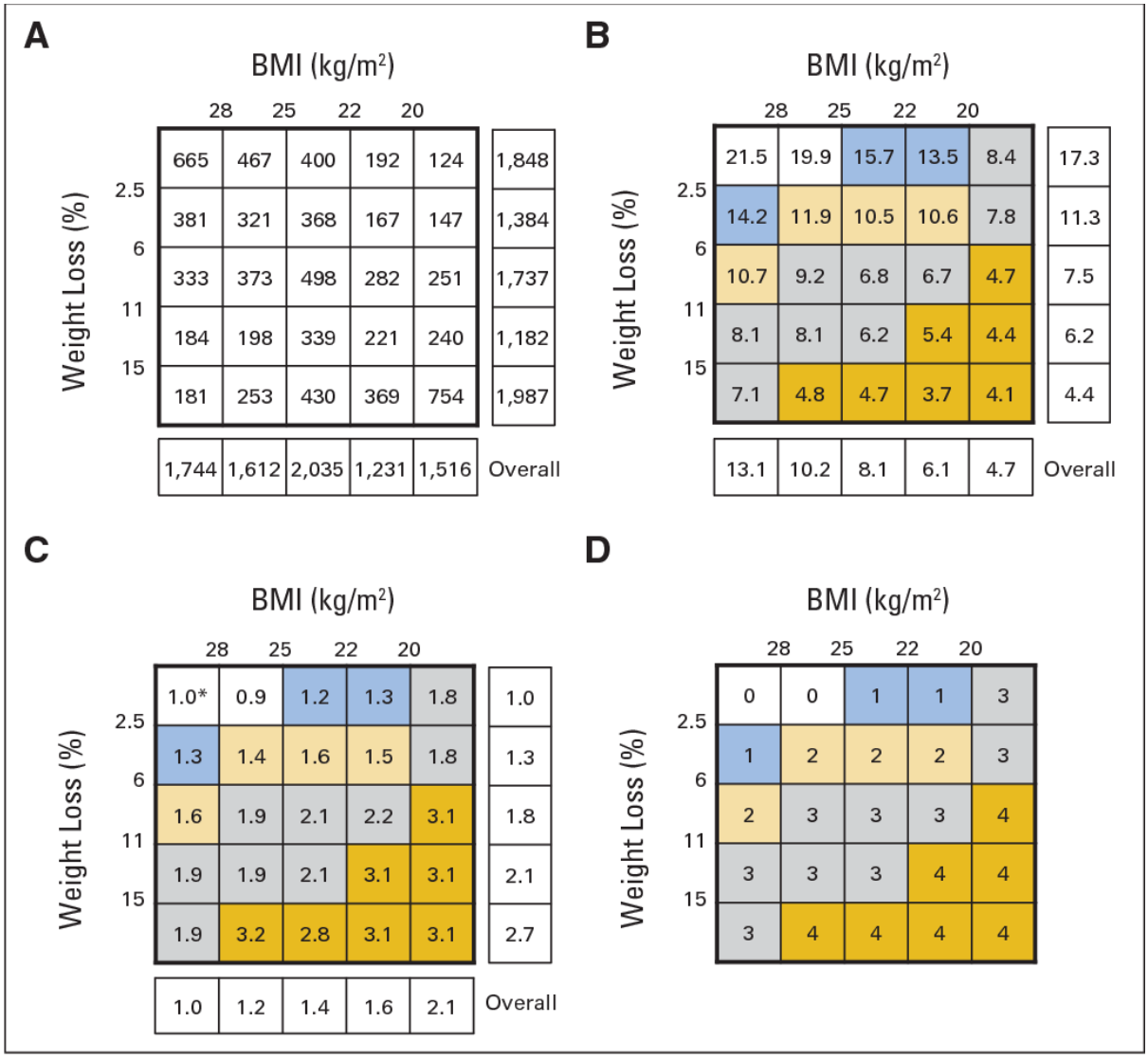


Figure 13 Diagnostic criteria for cancer associated weight loss (222, reproduced with permission from JCO)

Risk of reduced survival as a function of BMI and percentage weight loss. Panel A represents number of patients, panel B is median survival in months and panel C is the unadjusted estimated hazard ratios. Panel D represents the staging system. Colours represent grading from 0 to 4. Grade 4 is of value in identifying individuals whose expected survival is too short to merit specific treatment plans or interventions and should therefore be taken into account when risk assessing patients with cancer-associated weight loss. Finding that degree of weight loss affects survival supports the suggestion that cachexia has degrees of severity, even within the distinct phases of the syndrome.

1.11 Cachexia clinical trials

Many of the previous cachexia trials have been discussed alongside their targeted mediators, but there are a few which merit more attention (summarised in figures 14 and 15). Over the last few years there have been five phase 3 randomised, double blind placebo-controlled trials. Most notably the potential of Ghrelin as a treatment for cancer cachexia was investigated in the ROMANA I and II trials, and the ROMANA 3 safety extension study (93,225). Anamorelin, an oral and specific ghrelin receptor agonist, was given to patients with advanced non-small cell lung cancer and cachexia. Lean body mass increased in patients taking Anamorelin compared with placebo in all trials, as did body weight and anorexia-cachexia related symptoms. However, the trials were unable to demonstrate differences in handgrip strength as the functional co-primary endpoint. Food intake was not recorded, and it is therefore unclear as to whether an increase in appetite and nutritional status led to the observed changes in body mass. Besides anamorelin, the other novel Ghrelin agent macimorelin (NCT01614990) is currently under investigation, the results of which are expected in 2020 (226).

Other emerging anabolic agents are the selective androgen receptor modulators (SARMs). These drugs, most notably Enobosarm which was investigated in the POWER 1 and 2 trials are a new class of non-steroidal (227,228). SARMs are unique in their ability to increase muscle mass without

anabolic side effects. The POWER trials were randomised, double-blinded, placebo-controlled trials in patients with advanced lung cancer who had at least 2% weight loss in the previous 2 months. They both involved 3mg of Enobosarm or placebo daily at the start of chemotherapy (Platinum + Taxane in POWER 1 and Platinum + Non-Taxane in POWER 2). Unpublished results have shown a significant increase in lean body mass (LBM) but no increase in mean stair climb power (228).

Previous therapies for muscle wasting in cancer have focused on improving appetite, reducing inflammation and modifying muscle protein anabolism and catabolism. No single agent has yet been shown to be able to do this, highlighting the need for an integrated approach. This has led to the development of the MENAC trials (multimodal-exercise, nutrition and anti-inflammatory medication for cachexia) for palliative lung and pancreatic cancer patients (229,230). The pre-MENAC trial showed that a multimodal intervention in cachexia may improve weight, leading to the phase III MENAC trial [NCT01419145] (231). It consists of non-steroidal anti-inflammatories, eicosapentaenoic acid, a physical exercise programme using resistance and aerobic training, as well as dietary counselling and oral nutritional supplements versus standard cancer care. The trial is ongoing.

Figure 14 Targets for previously trialled cachexia drugs Drugs are highlighted in orange boxes alongside their targets [Adapted from (232)]

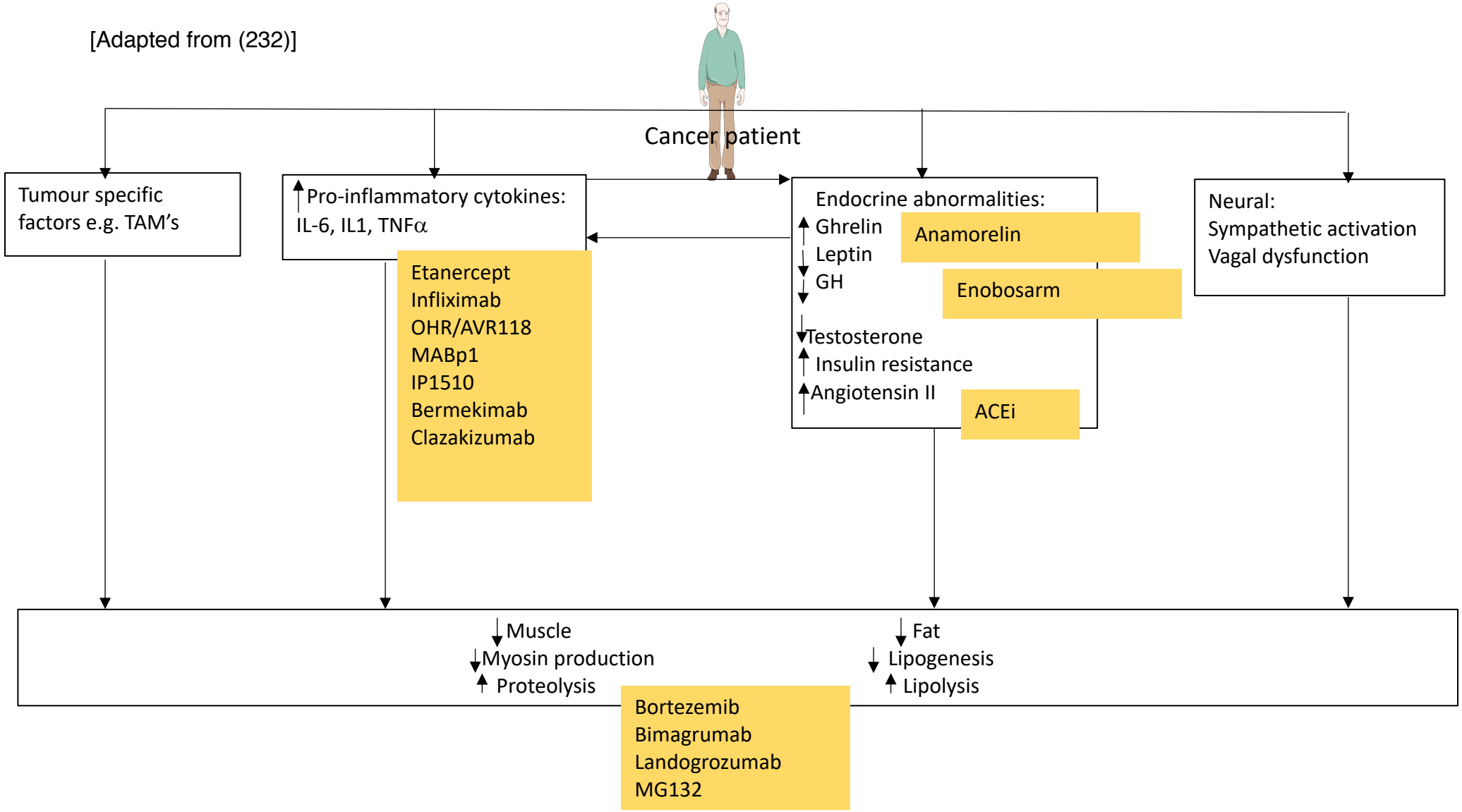
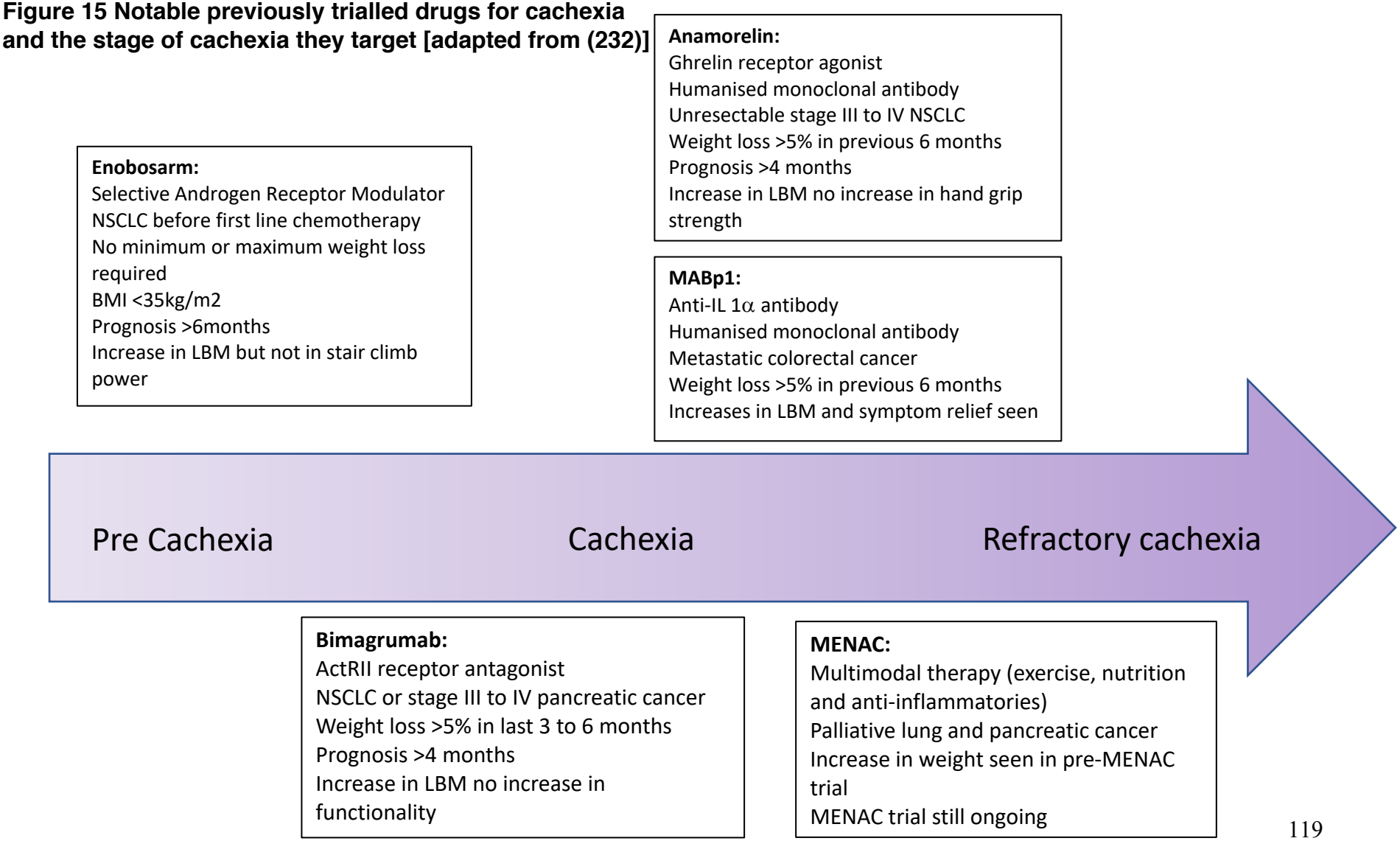


Figure 15 Notable previously trialled drugs for cachexia and the stage of cachexia they target [adapted from (232)]



To date no clinical trials have been considered wholly successful in the treatment or prevention of cancer cachexia. This is in part due to difficulties in assessing cachexia diagnosis but also due to a lack of clear consensus on how to measure primary end points of change in LBM and physical activity. Recent trials have used various outcome measures including stair climbing, hand grip strength, 6-minute walk test and the timed up and go, and a consensus needs to be made on which is the most appropriate choice to inform future trial design

This introductory overview has highlighted that muscle wasting in cancer is multifactorial and complex. It is underpinned by hypercatabolism, a net loss of energy, and systemic inflammation. Although its effects are most devastating later in the disease process, molecular changes are likely to begin very early on. Previous therapeutic targets have been promising in preclinical models but have not been fully efficacious in clinical trials. The availability of both promising animal and clinical studies do, however, highlight that treatments need to be targeted and supported by multi-modal approaches. Research needs to focus on the link between pre-clinical and clinical models to unpick the mechanisms involved and develop standardised methods and outcomes for future clinical trials.

1.12 Research questions

The introductory chapter has attempted to provide an overview of cancer cachexia with a particular focus on recently discovered mediators which may lend themselves as future therapeutic targets. In particular, adipose wasting and tissue cross-talk may have important undiscovered effects on patient outcomes. There still remains difficulty in assessing patients due to the lack of a cachexia-related biomarker and increasing rates of obesity since the consensus definition was introduced. Therefore, this thesis is targeted at evaluation of patient body composition and signalling pathways within cachectic human plasma, muscle and fat. A number of studies have been undertaken with the main questions being asked summarised below:

1. Is it possible to use one single screening tool to assess cachexia, sarcopenia and malnutrition? If so is it able to distinguish between the three conditions?
2. What is the relationship between CT derived measures of body composition and age and sex in patients with oesophago-gastric cancer?
3. Are abnormalities in the neuromuscular junction responsible for the muscle wasting observed in cancer cachexia?
4. What are the transcriptomic differences in visceral and subcutaneous adipose depots in patients with cancer cachexia and healthy controls?

5. Are we able to identify potential plasma biomarkers of cancer cachexia through metabolomics analyses?

CHAPTER 2

GENERAL METHODS

2.0 General Methods

This next section describes methods that were common to all studies included in this thesis. It details patient recruitment, anthropometric measures, body composition analysis and tissue sampling. Functional and QoL assessment was also performed as part of a wider biobank collection and as such is briefly described, however this data was not utilised in the studies that made up this thesis.

2.1 Participant groups

Patient data were drawn from three sources. An existing and continuing biobank (created through a funded collaboration with Novartis) which contains >250 samples of rectus muscle, subcutaneous and visceral fat, urine and plasma from robustly phenotyped patients, a previous CRUK biomarker study, and the prospectively collected tissue for the neuromuscular junction study.

1. Muscle wasting in cancer study: An ongoing study of healthy controls, vascular and cancer patients at the Royal Infirmary of Edinburgh (RIE). Patients underwent CT, functional assessment, QoL assessment, blood, fat, urine and muscle sampling at the time of their operations
2. CRUK cancer biomarker study: A previous study of patients with UGI cancer at the RIE who underwent functional assessment, MRI quadriceps, CT scanning, blood, urine and muscle sampling. (n=121 patients with cancer)

3. NMJ study: An ongoing study at the RIE assessing the NMJ involving UGI cancer and vascular patients. Patients underwent CT, functional assessment, QoL assessment, blood and muscle sampling at the time of their operations

2.2 Participant recruitment

2.2.1 Cancer patient recruitment

All cancer patients who were prospectively recruited attended the Surgical Department of the RIE. Staging was carried out according to the American Joint Committee on Cancer (AJCC)/Union Internationale Contre le Cancer (UICC) systems. Ethical approval for the study was obtained from the South East Scotland Research Ethics Committee, co-sponsored by the University of Edinburgh and National Health Service (NHS) Lothian Health Board (IRAS ID 190214). In accordance with the Helsinki Declaration, all patients provided written and informed consent prior to surgery. Patients who were suitable to undergo potentially curative surgery were identified at the regional UGI cancer multi-disciplinary team meetings (process summarised in figure 16). They were approached at their routine pre-operative clinic appointment and patient information sheets were given (appendix 1). QoL (appendix 2) and the timed-up-and-go (TUG) and blood tests were performed at this appointment.

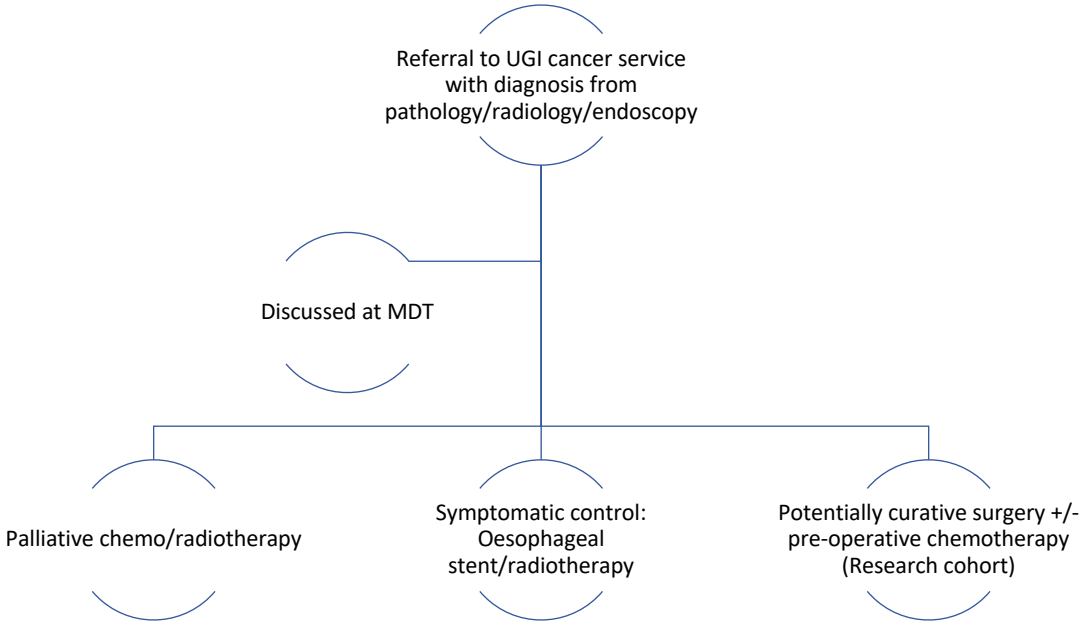


Figure 16 Cancer patient recruitment process

Patients with a suspected or confirmed diagnosis of UGI cancer were referred to the central MDT meeting (comprising surgeons, radiologists, pathologists, nurse specialists, physicians, oncologists, and dieticians). A consensus decision on treatment was then made. Patients included in this thesis were recruited from the curative surgery cohort. Abbreviations: MDT, multi-disciplinary team; UGI, upper gastrointestinal

2.2.2 Control patient recruitment

Patients undergoing elective surgery for aortic aneurysmal or occlusive disease were approached once admitted to the vascular unit the day prior to their planned surgery. Vascular patients were chosen as they were all undergoing laparotomy meaning tissue was easily accessible. They also form an interesting group of patients who often have low muscularity despite not suffering from cancer. Healthy patients who were undergoing laparoscopic surgery for live renal transplant donation were approached at their routine pre-operative assessment appointment. Baseline blood tests were taken routinely. Written informed consent was obtained. QoL and TUG test were performed at the same meeting.

2.3 Anthropometric measurements

2.3.1 Height, weight and weight changes

Body weight was measured with patients lightly clothed using a beam scale (Seca, UK). Height was measured using a standard wall mounted measure. Weight change was calculated as the percentage difference between stable pre-morbid weight (self-reported by the patient) and the weight at assessment. There is evidence that self-reported weight is reliable, with height and weight measurements performed by physicians and nurses being shown to have a 10% margin of error (233).

2.3.2 Body mass index (BMI)

BMI was calculated according to the formula:

BMI (kg/m^2) = weight (kg)/height $(m)^2$

2.4 Muscle mass measurement

2.4.1 CT scan identification and location

Patients routine diagnostic and staging CT were analysed for muscle mass assessment. Recent CTs performed at the RIE were easily identified from the electronic clinical record (TrakCare, InterSystems Corporation, Cambridge Massachusetts). CTs could then be found on the PACS (Picture Archiving and Communications System) electronic x-ray viewing system. Scans performed at peripheral hospitals could be located on the national PACS archive.

2.4.2 Slice selection and preparation

Cross sectional images were located at the level of the third lumbar vertebra (L3) on abdominal CT. L3 was identified by locating the lowest thoracic vertebra (the lowest vertebral body with ribs) and counting vertebra in a caudal direction. The slice with both transverse processes visible was taken as the first slice. Cross sectional images were saved in the DICOM format.

2.4.3 Tissue segmentation

Muscle cross sectional area (CSA) was measured with SliceOmatic V4.3 software (Tomovision, Canada) and the Alberta protocol from the ABACS

software (Vorioni, Canada) according to a previously established protocol. Tissue segmentation was performed using Hounsfield unit ranges -29 to +150 for muscle, -190 to -30 for subcutaneous adipose tissue, -190 to -30 for intramuscular adipose tissue, -150 to -50 for visceral adipose tissue. Each tissue type was manually drawn out if required and abdominal viscera excluded. Muscle groups included were Quadratus lumborum, Erector spinae, Psoas major and minor, Internal and External abdominal oblique, Transversus and Rectus Abdominus. The software outputted data as a CSA in cm^2 for each tissue which was then converted into the skeletal muscle index (SMI) as described. below. An example of the CT analysis undertaken is shown in figure 17.

SMI was calculated as the ratio of lumbar skeletal muscle area to height and was defined according to Martin et al: SMI <43cm^2/m^2 for men with a BMI <25, <53 for men with a BMI ≥ 25 and <41cm^2/m^2 for women (27). Subcutaneous and visceral adipose tissue was indexed for height (SATI and VATI). Low SATI was defined as <50cm^2/m^2 in males and <42cm^2/m^2 in females. Low VATI was defined as <52.9cm^2/m^2 in males and <51.5cm^2/m^2 in females (234). Total adipose tissue index (TATI) was defined as the sum of VATI + SATI. High TATI was considered to be ≥107.7cm^2/m^2 in men and ≥102.2cm^2/m^2 in women (223). Intramuscular fat was described in terms of cross-sectional area (CSA). Sarcopenic obesity was defined as sarcopenia in the presence of BMI ≥30 kg/m^2 (221).

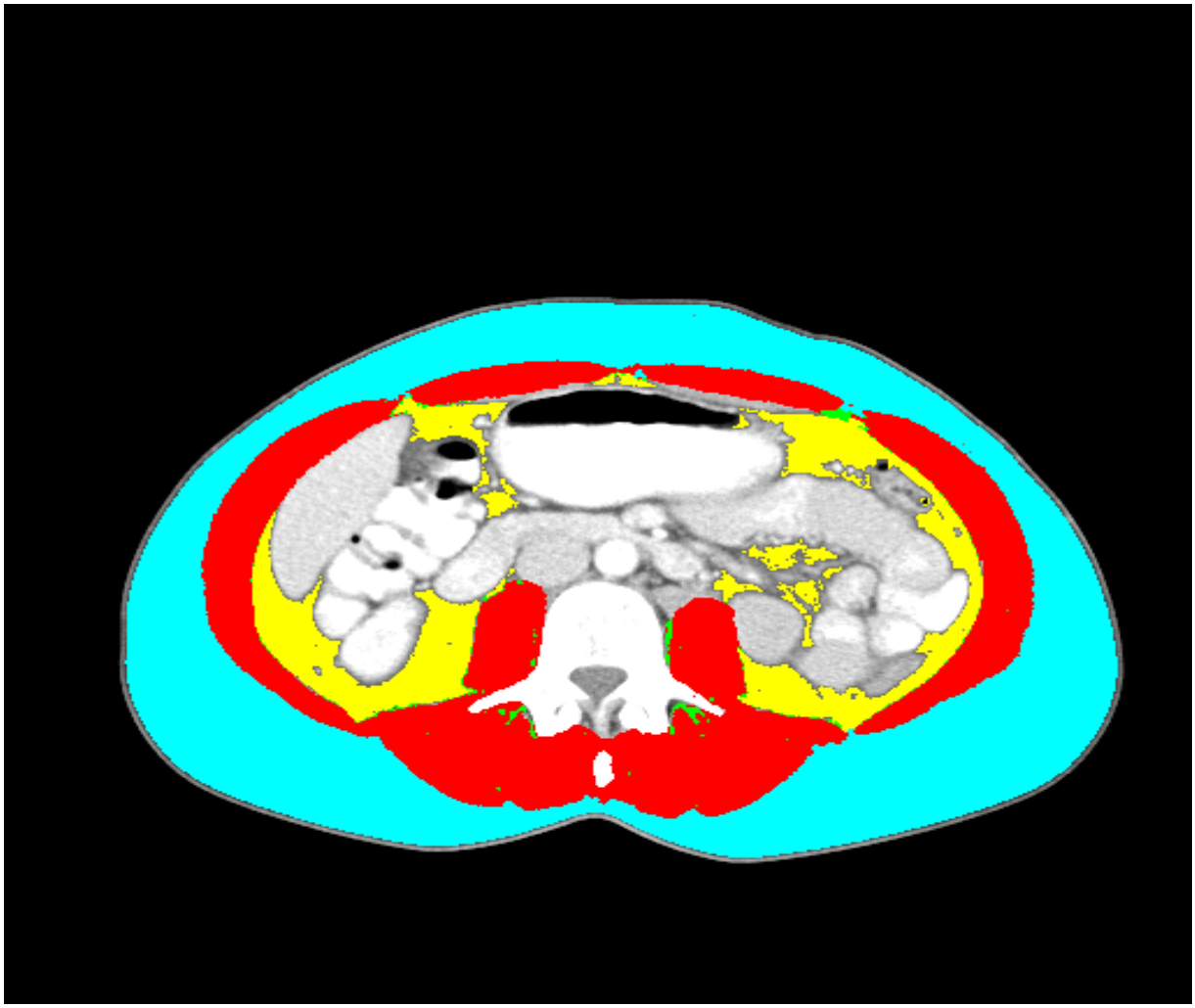


Figure 17 Cross sectional CT image

Image take at L3 showing demarcation of skeletal muscle and adipose tissue using Slice-o-matic and ABACS software.

Light blue – subcutaneous fat
Red – muscle
Green – intramuscular fat
Yellow – intra abdominal fat

2.5 Functional measures

2.5.1 Sit to Stand times (STS)

STS times were measured by a single observer using a digital stopwatch. The patient began seated with their arms by their side and feet in front of the chair. Once instructed that the test had begun, they were to rise from the chair. The test stopped when they were in the upright position. The STS test is associated with coefficient of variation for repeated tests of between 6.8% (healthy volunteers) and 14% (elderly) (235).

2.5.2 Timed up and go (TUG)

TUG was measured in conjunction with the STS test, and was timed by one observer using a digital stopwatch. The participant undertook the STS test and then were instructed to walk at their normal pace for three meters and then return to the chair and sit down again. The TUG test is recognised as a reliable and reproducible test with an inter-rater reliability intra-class correlation coefficient (ICC) of .99 and an intra-rater ICC of .98 (236).

2.6 Quality of life assessment

2.6.1 Karnofsky performance score (KPS)

KPS was assessed in each patient by one observer. Appendix 2 outlines the scoring system.

2.6.2 European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire Core 30 (EORTC QLQ-C30)

EORTC QLQ C-30 was assessed in all patients and results analysed using linearised scores. The questionnaire is outlined in appendix 2.

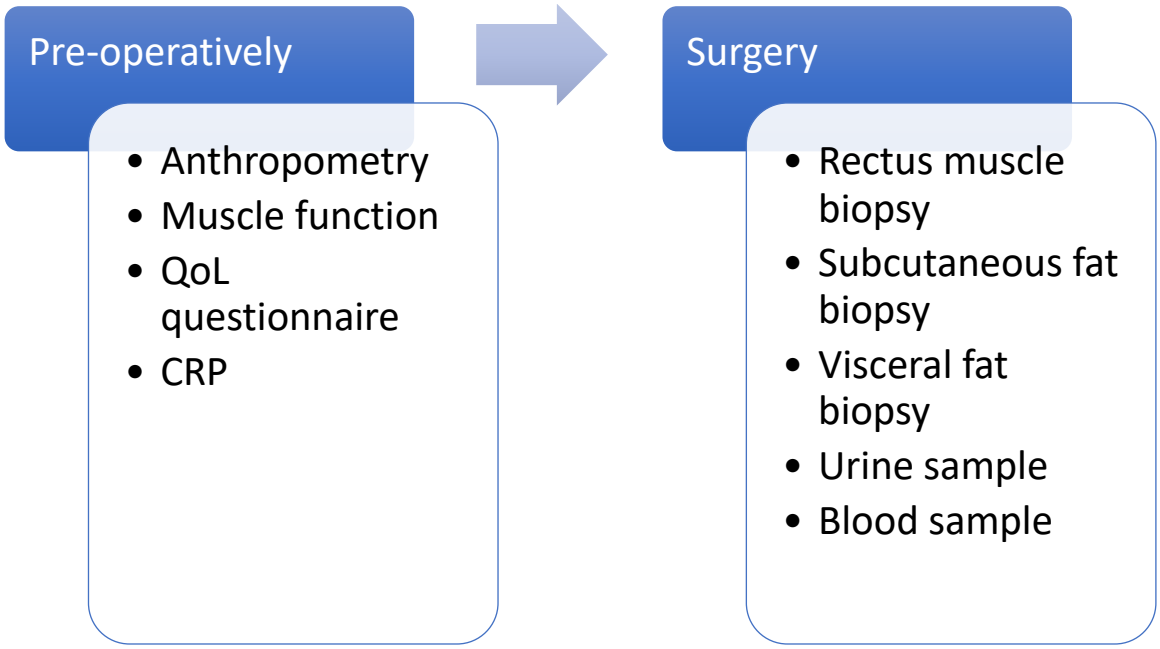


Figure 18 Data and samples collected for each patient

Each patient underwent pre-operative functional and QoL assessment as well as having blood taken for inflammatory markers. Plasma was sampled at the induction of anaesthesia. Tissue sampling was performed intra-operatively.

2.7 Tissue sampling

2.7.1 Muscle wasting in cancer study

All biopsies were taken at the start of open or laparoscopic abdominal surgery during the initial incision.

Muscle: Approximately 1cm^2 biopsies of rectus abdominus were taken using sharp dissection. Samples were cleaned of fat/blood/fibrous tissue, placed in a tube (NUNC Cryotubes, Sigma-Aldrich Co. LLC) snap frozen, and stored at –80°C. Three sections of the muscle were treated with optical cutting temperature (OCT) and lowered into 5mls of cooled isopentane solvent (–190°C) prior to storage at –80°C. Specific details of muscle taken for the NMJ study are described in the corresponding chapter.

Fat: Approximately 1cm^2 biopsy of subcutaneous fat was taken from all patients. Visceral omentum was sampled from cancer and vascular patients and perinephric fat from controls. Omental fat was sampled at the beginning of the operation whereas perinephric fat was sampled once the donor kidney had been removed. Samples were snap frozen in liquid nitrogen and stored at -80°C.

Urine: Urine was taken from the patient at the time of general anaesthesia following an overnight fast. It was collected in a sterile container, divided into 2 ml aliquots, and snap frozen in liquid nitrogen, prior to storage at -80^{o}C

Plasma samples: Venous blood samples were taken using 2 x 9ml serum Monovette tubes and 1x EDTA tube (Sarstedt,Nümbrecht, Germany). Samples were centrifuged at 4°C at 15 000 x g for 15 mins. Plasma was removed with a pasteur pipette and divided into 2ml aliquots. These were frozen at -80°C prior to processing.

2.7.2 Blood measures

All pre-operative samples were processed by the Department of Clinical Chemistry, RIE (fully accredited by Clinical Pathology Accreditation Ltd, UK) CRP was measured by enzyme linked immunosorbent assay (ELISA) (Ely, UK).

Further detailed methods pertaining to each study are described in more detail in the relevant chapters.

CHARACTERISATION OF CANCER CACHEXIA

Chapters 3 and 4

CHAPTER 3

VALIDATED SCREENING TOOLS FOR THE ASSESSMENT OF CACHEXIA, SARCOPENIA AND MALNUTRITION: A SYSTEMATIC REVIEW

3.1 Overview of chapter

The first chapter in this thesis focusses on the ability to screen for cachexia. Malnutrition and sarcopenia also present with unintentional weight loss and so the ability to pick out the underlying condition would aid in targeting patients for selection into clinical trials as well as instigating appropriate treatments.

3.1.1 Objectives

To systematically review validated screening tools for cachexia, sarcopenia and malnutrition in adults and, if a combined tool is absent, make suggestions for the generation of a novel screening tool.

3.1.2 Methods

A systematic search was performed in Ovid MEDLINE, EMBASE, CINAHL (Cumulative index to Nursing and Allied Health Literature) and Web of Science. Two reviewers performed data extraction independently. Each tool was judged for validity against a reference method. Psychometric evaluation was performed as was appraisal of the tools ability to assess the patient against consensus definitions.

3.1.3 Results

Thirty-eight studies described 22 validated screening tools. The Cachexia score (CASCO) was the only validated screening tool for cachexia; performing well against the consensus definition. Two tools assessed sarcopenia (the

Short Portable Sarcopenia Measure [SPSM] and the SARC-F); scoring well against the 1998 Baumgartner definition. The SPSM required large amounts of equipment and the SARC-F had a low sensitivity. Nineteen tools screened for malnutrition. The 3 minute nutrition score (3 MinNS) scored best meeting consensus definition criteria (ESPEN) and also having sensitivities and specificities >80%. No tool contained all the currently accepted components to screen for all three conditions. Only three tools were measured against cross-sectional imaging, a clinical tool that is gaining wider interest in body composition analysis.

3.1.4 Conclusion

No one validated screening tool can be implemented for the simultaneous assessment of cachexia, sarcopenia and malnutrition. The development of a tool that encompasses consensus definition criteria and directs clinicians towards the underlying diagnosis would be optimal to target treatment and improve outcomes. Proposed is a tool that incorporates a stepwise assessment of nutritional status; oral intake; disease status; age; muscle mass/function; and metabolic derangement.

3.2 Introduction

Unintentional weight loss (UWL) as a form of nutritional depletion is commonly seen in ageing, cancer and many chronic diseases. As discussed in the introductory section the main subtypes can be categorised into three primary syndromes: cachexia, age-related sarcopenia and malnutrition; however, it is not clear whether existing screening tools are able to distinguish between these three conditions. This is due in part to the complex overlap between them. Loss of muscle mass is a key feature in both cachexia and sarcopenia but patients with sarcopenia are not necessarily cachectic. As we have seen there are many definitions for each condition, with nutritional depletion playing a part in each, therefore making it difficult to separate them out (28,174,237,238). These conditions are also often not noticed in their earlier phases but do become apparent following a critical event or development of disability (239).

More than 70 nutritional screening tools for use in hospitals have been developed to facilitate easy screening or assessment of a patient's nutritional status or to predict poor clinical outcomes related to UWL. Despite increasing research, there appears to be a lack of a practical and implementable clinical screening tool to support diagnosis (240). In the general community, the European Society for Clinical Nutrition and Metabolism (ESPEN) endorses the use of Malnutrition Screening Tool (MUST) (240,241), and Nutritional Risk Screening (NRS-2002) (242) and the Mini Nutritional Assessment – Short

Form (MNA-SF) for the elderly (243,244). Some tools claim to have been developed to screen in specific target groups however, there are currently no disease specific recommendations. There is no international consensus on a single 'best tool' to identify all three syndromes across populations. The use of different tools in different studies makes drawing any conclusions about their comparison and meta-analyses difficult.

Current diagnostic methods for sarcopenia and cachexia include the measurement of total body potassium and water, assessment of body anthropometry using either body mass index (BMI) or estimated weight loss, or by direct assessment of muscle and fat mass using dual-energy X-Ray absorptiometry (DXA), bioelectrical impedance analysis (BIA), CT or MRI scanning. Whilst the latter two radiographic modalities are accurate, they are impractical, expensive and some expose the patient to ionizing radiation. This diagnostic approach to detect the presence of sarcopenia is time consuming, expensive and requires highly specialised equipment (245). Therefore, a screening tool that is implementable in a larger population that allows for early detection is important. This approach would highlight the potential for further assessment with early biomarkers, thus allowing prophylactic intervention in malnutrition and driving further research in sarcopenia and cachexia.

This chapter aims to systematically review validated screening tools for the general adult population to enable clinicians to distinguish between the three

syndromes. The specific strengths and limitations of each tool were assessed, as was the appropriateness of the validation population. Through psychometric evaluation and assessment of the tools against the agreed consensus definitions, we also investigated if any one single tool could be used for the simultaneous assessment of all three.

3.3 Methods

Methods for conducting systematic reviews of the effectiveness of interventions have been well described. In accordance with PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) (246) guidelines we applied the principles to systematically reviewing validated screening tools used in the assessment of cachexia, sarcopenia and malnutrition.

3.3.1 Literature review

A systematic search was performed on 7th August 2018 in Ovid MEDLINE (1946-2017), EMBASE (1974-2017), CINAHL (Cumulative index to Nursing and Allied Health Literature) and Web of Science. Relevant articles were identified by title and abstract. Reference lists of review articles were also hand searched. Double data extraction was performed by two reviewers independently to ensure consistency. Any disagreements were settled by a third reviewer.

The basic search strategy was “Sarcopenia” OR “Cachexia” OR “Malnutrition” AND “screening” AND “validation study” using MeSH terms and keywords appropriate to each database. No language restriction was imposed. The search was designed to be broad to ensure all validated tools were identified. There were no disease specific or language limits.

3.3.2 Inclusion and exclusion criteria

Studies were included if they had developed a screening tool which had been validated for the screening of either cachexia, sarcopenia or malnutrition in adults (Table 3). Disease specific tools were included. Papers were excluded if the tools had not been validated or if they assessed malnutrition in children or obesity in adults. Studies which described modified versions of pre-existing tools were also excluded as this was out with the scope of this review. It was intended that studies that included less than 25 patients should be excluded as they were unlikely to yield robust, generalisable psychometric results, however no studies with numbers smaller than this were found.

Types of participants	
Adults (>18 years) undergoing routine screening for cachexia, sarcopenia, or malnutrition	Includes patients with advanced cancer, end stage cardiac, renal and liver disease
Types of tools	
Validated, quantitative measurements of cachexia, sarcopenia or malnutrition	Tools developed for clinical or research purposes. Completed by health care professionals
Psychometric evaluation	**Demonstration of at least 2 criteria:**
Content Validity	Breadth of scope of tool; to what extent does it appear to capture the relevant aspects of unintentional weight loss; are there gaps?
Construct validity, including convergent validity, discriminant validity	How well the tool relates to other measures of the same construct; lack of correlation with dissimilar or unrelated constructs or variables
Test-retest reliability	How consistent an individual's scores are over a defined time-period presuming weight stays constant
Internal consistency	How closely related are the different items in the tool?
Responsiveness	Ability to detect clinically meaningful change for individuals
Factor analysis	For a tool comprising several items, a way of grouping them into factors which may tap into a particular construct
Criterion validity	A shortened version of a scale, concurrent validity with the longer version which has been validated

Table 3 Systematic review Inclusion criteria

3.3.3 Assessment of Validity

Studies had to have evaluation of at least two of the following psychometric characteristics:

- Content validity
- Construct validity e.g. including convergent validity, discriminant validity
- Test-retest reliability
- Internal consistency
- Responsiveness
- Factor analysis
- Criterion validity

Primary criteria used to evaluate the tools were construct validity and responsiveness.

3.3.4 Criterion and construct validity, reference method

Studying the validity of a tool usually compares to a gold standard. Although many research groups are now using cross sectional imaging to investigate UWL there is currently the absence of a perfect gold standard. Studies used different reference methods to validate their tools e.g. DXA and assessment by a health professional. The tools SGA and MNA are the tools currently recognised as the industry standard and were therefore considered valid

references (238,240). The term criterion validity was used for these comparisons.

Less valid reference methods including the use of other screening tools and blood tests for example albumin which can be influenced by other factors including inflammation and acute disease were included as many research groups vary in their opinion on the optimal reference method (247). These comparisons were termed construct validity.

3.3.5 Predictive validity

Predictive validity was assessed as the ability of the tool to predict the probability of a better or worse clinical outcome due to nutritional risk.

3.3.6 Diagnostic criteria

Tools were also assessed for their ability to identify the risk of cachexia, sarcopenia or malnutrition by comparison of their components against the components of each set of chosen diagnostic criteria (Table 4).

Syndrome	Diagnostic criteria
Cachexia	Weight loss greater than 5%, or weight loss greater than 2% in individuals already showing depletion according to current bodyweight and height (body-mass index [BMI] <20 kg/m^2) or skeletal muscle mass (sarcopenia) (28)
Sarcopenia	Loss of function – 6-minute walk < 400m OR gait speed <1.0m/s Muscle mass – low appendicular lean mass/height (2 standard deviations below the mean diagnostic on DXA) (174,237)
Malnutrition	Protein/energy deficiency - risk indicated by low BMI <18.5 kg/m^2 OR weight loss >10% (indefinite time)/5% over last 3 months AND BMI <20 (if <70 years)/ <22 (if>70 years) or FFMI < 15 and 17 kg/m^2 in men and women respectively (238)

Table 4 Summary of proposed diagnostic criteria for identification of cachexia, sarcopenia and malnutrition

FFMI (Fat-free mass index)

3.3.7 Assessment of bias

Assessment of bias was made using a form of the Newcastle-Ottawa scale adapted for cross-sectional studies (248). Each study was scored out of 10 and a study with a score of <5 was considered to be at high risk of bias. Full details of the scoring used can be found in the appendix.

3.3.8 Secondary criteria

Secondary criteria included face validity, development and content validity, factor analysis, test-retest reliability, internal consistency, respondent and

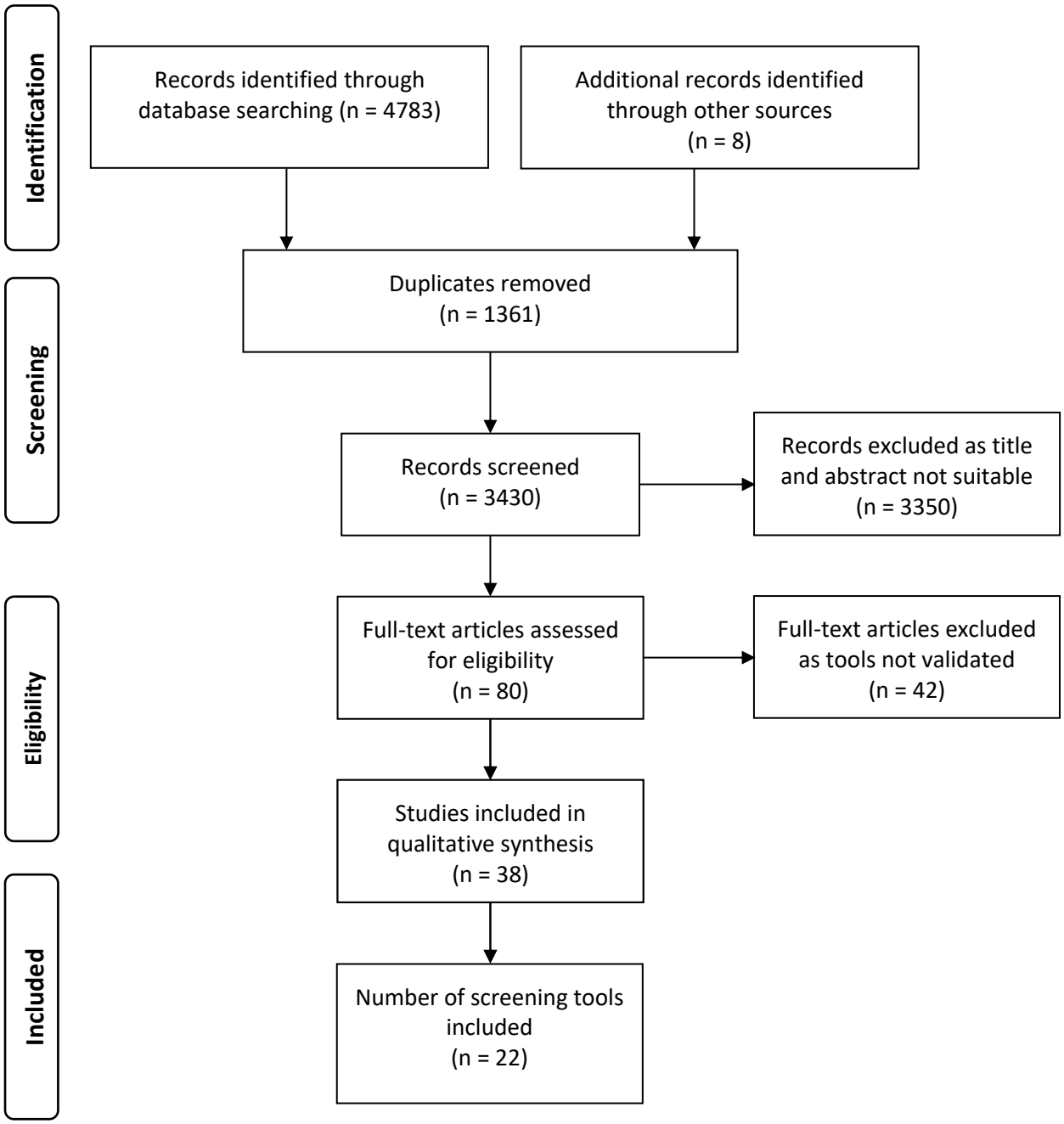
administrative burden (the time and effort required to complete the tool). These are also summarised in table 3. Data were extracted concerning the study participants, the tool used and psychometric evaluations (Inclusion criteria Table 3). Assessment of sensitivity and specificity was made. A value >80% was considered good, 60-80% fair and <60% poor. Agreement was also assessed as: 0.9-1 = excellent, 0.80-.90 = good, 0.60-0.80 = fair and <0.60 = poor.

3.4 Results

3.4.1 Principal findings

Thirty-eight studies were included which described the validation of 22 screening tools. The majority of papers were excluded as they described un-validated tools. This is summarised in figure 19.

Figure 19 PRISMA flow diagram to show source of studies included in the review (246)



The Cachexia score (CASCO) was the only screening tool for cachexia that had been validated. It performed well against diagnostic criteria (28), but sensitivities and specificities were not recorded. Only two tools assessed sarcopenia (the Short Portable Sarcopenia Measure [SPSM] and the SARC-F) and scored well against the agreed definition (Baumgartner 1998). However, the SPSM required a large amount of equipment and the SARC-F had a very low sensitivity. Both were validated for use in the outpatient setting. Nineteen tools screened for malnutrition. The 3 minute nutrition score (3 MinNS) proved to be the best, scoring well against the consensus definition (ESPEN) as well as having sensitivities and specificities >80%. There was no one validated tool which adequately screened for all three conditions. A critical appraisal of all tools can be found in table 5.

Table 5 Critical appraisal of tools to measure unintentional weight loss

	Author	Tool	Description	Validation population	Validation reference	Strengths	Limitations
Sarcopenia	Woo et al, 2014 (249)	SARC-F	A questionnaire regarding ability to carry a heavy load, walking, rising from a chair, climbing stairs and falls frequency	Community dwelling Chinese (n=4000)	3 consensus definitions of sarcopenia	Not dependent on cut off values	No assessment of muscle mass, not validated in hospital populations
	Miller et al, 2009 (250)	SPSM	Portable measure that combines estimates of muscle quantity and function into a single scale	Community dwelling African Americans (n=998)	DXA	Portable	Time consuming, equipment dependent, muscle mass not measured
Cachexia	Argiles et al, 2017 (251)	CASCO	Score to classify cachectic patients into three different groups. Includes five components: body weight loss & composition, inflammation/metabolic disturbances/immunosuppression, physical performance, anorexia and quality of life	Cancer patients (n=186)	Assessment by oncologist	Encompasses all diagnostic criteria	Involves many questions and measurements, does not include questions on disease state
Malnutrition	Weekes et al, 2004 (252)	BAPEN	Tool based on four nutritional parameters (weight, height, recent unintentional weight loss and appetite)	Acute medical and elderly care wards (n=100)	Dietician	Quick and easy	Percentage weight loss not quantified
	Mimiram et al, 2011 (253)	BNST	Score based on UWL, unintentional eating loss and being unable to eat for >5 days	Medical and surgical (n=446)	Dietician	Easily completed by nursing staff	Low importance given to amount of weight loss
	Laporte et al, 2015 (254)	CNST	Tool containing two items: Weight loss and decreased food intake	Medical and surgical (n=150)	SGA	Very brief, can be completed by non-trained rater	Assessed on admission only. Validity of re-screening unknown
	Ignacio et al, 2005 (255)	CONUT	Evaluates nutrition using albumin, cholesterol and lymphocyte count. Automated system	Medical and surgical inpatients (n=53)	SGA	Simple, automated	Markers vary depending on disease state, only done on patients who have bloods taken

	Guerra et al, 2017 (256)	EDC	Screening tool based on ESPEN criteria for diagnosis malnutrition	Medical and surgical inpatients (n=632)	PG-SGA	Includes FFM assessment	Very low sensitivity
	Abd-El-Gawad et al, 2014 (257)	GNRI	Modified nutritional risk index for geriatric patients (based on albumin, current and previous weight)	Acute geriatrics ward (n=131)	MNA	Good prognosticator, does not require capacity	Diseases associated with high mortality or hypoalbuminaemia excluded
	Tammam et al, 2009 (258)	INSYST	Two-tiered tool – first is a simple pre-screen aiming to establish if malnourished, second provides a more detailed evaluation	Medical, surgical and oncological inpatients (n=61)	MUST and MNA	Doesn't require height and BMI, quick and easy	Ease of completing dependent on patient's cognitive state
	Ferguson et al, 1999 (259) Isenring et al, 2006 (260) Neelemaat et al, 2011 (261) Nursal et al, 2005 (262) Young et al, 2013 (263) Wu et al, 2012 (264) Bhuachalla et al, 2018 (265) Leipold et al, 2018 (266)	MST	Two questions regarding appetite and unintentional weight loss	Medical and surgical inpatients (n=408) Oncology outpatients (n=51) Acute hospitalised (n=193) Medical and surgical inpatients (n=2211) Elderly medical inpatients (n=134) Elderly inpatients (n=157) Oncology patients (n=725) Rehabilitation patients (n=160)	SGA PG-SGA Malnutrition definition CT	Very quick, does not require calculations	Non-specific
	Kim et al, 2011 (267)	MSTC	Tool based on intake change, weight loss, ECOG performance status and BMI	Oncology inpatients (n=1057)	PG-SGA	Cancer specific	Designed to be performed by dieticians, not nurses
	Boleo-Tome et al, 2012 (268) Leistra et al, 2013 (269) Sharma et al, 2017 (270) Neelemaat et al, 2011 (261) Kyle et al, 2006 (271) Young et al, 2013 (263) Almedia et al, 2012 (272) Velasco et al, 2011 (273)	MUST	Five step tool including BMI, unplanned weight loss and presence of acute disease	Oncology inpatients (n=450) Medical and surgical outpatients (n=2236) Acute medical inpatients (n=132) Elderly inpatients (n=198) Medical and surgical (n=995) Medical inpatients (n=134) Surgical inpatients (n=300) Medical and surgical (n=400) Oncology patients (n=725)	PG-SGA Malnutrition definition CT	Quick, easy	Does not pick up patients with normal BMI who are malnourished, UWL reported by patients is subjective

	Bhuachalla et al, 2018 (265)	NRI	Derived from serum albumin concentration and ratio of usual to present weight	Peritoneal dialysis patients (n=283) Colorectal cancer (n=52) Oncology patients (n=725)	SGA CT	Assesses dialysis patients at risk	Relies on previous weight – limited use with changes in fluid status
	Prasad et al, 2012 (274) Faramarzi et al, 2013 (275) Bhuachalla et al, 2018 (265)	NRS-2002	Tool containing nutritional components of the MUST along with disease severity	Elderly inpatients (n=198) Medical and surgical (n=995) Elderly medical patients (n=134) Surgical inpatients (n=300) Acute geriatrics ward (n=121) Medical and surgical (n=400)	Definition of malnutrition SGA	Includes disease severity therefore applicable in ITU	Ease of completing dependent on patient's cognitive state
	Neelemaat et al, 2011 (261) Kyle et al, 2006 (271) Young et al, 2013 (263) Almedia et al, 2012 (272) Bauer et al, 2005 (276) Velasco et al, 2011 (273)	NUFFE	Three-point ordinal scale with 15 items assessing weight loss, dietary history, appetite and general activity	Elderly care rehab ward (n=114)	MNA	Simple as lacks anthropometric measurements	Many confounding factors in questionnaire
	Soederhamn et al, 2002 (277) Duerksen et al, 2000 (278) Cooper et al, 2002 (279) Moriana et al, 2014 (280)	SGA	Assessment of nutritional status based on history and examination	Acute elderly care and elderly rehab (n=95) End stage renal disease (n=76) Medical and surgical inpatients (n=197)	Geriatric and internal medicine resident, Total body Nitrogen, Anthropometric and biochemical data	Current gold standard	Reproducibility less than in non-elderly, unable to predict severe malnutrition in ESRD, requires experienced operator to carry out
	Kruizenga et al, 2005 (281) Leistra et al, 2013 (269) Harada et al, 2017 (282) Neelemaat et al, 2011 (261) Young et al, 2013 (263)	SNAQ	26 questions related to eating and drinking difficulties, defecation, condition and pain	Medical, surgical and oncological inpatients (n=291) Medical and surgical outpatients (n=2236) Oncology outpatients undergoing chemotherapy (n=300) Medical and surgical inpatients (n=2211) Elderly medical inpatients (n=134)	Malnutrition criteria, CONUT	Corresponds to ESPEN criteria	High NPV, no outcome data

	Susetyowati et al, 2014 (283)	SNST	Six questions including weight loss, appetite and health status	Medical and surgical inpatients (n=495)	SGA	Can be done by non-trained staff	No anthropometric assessment, all subjective
	Wong et al, 2011 (284)	Spinal NST	Tool which assesses eight criteria including appetite, weight loss and level of spinal cord injury	Spinal cord injury patients (n=150)	Dietetic assessment	Disease specific	Requires specialised scales to measure paralysed patients
	Xia et al, 2016 (285)	R-NST	Nine questions assessing malnutrition risk/symptoms combined with albumin, CRP and urea	Renal inpatients (n=122)	SGA	Renal specific	Patients picked up for conditions other than malnutrition e.g. hyperkalaemia
	Lim et al, 2009 (286)	3-MinNS	Questionnaire based on diagnostic criteria for malnutrition and muscle wastage	Medical and surgical inpatients (n=818)	SGA	Quick and easy	Dependent on cognitive state

SPSM (Short Portable Sarcopenia Measure), CASCO (Cachexia Score), BAPEN (British Association for Parenteral and Enteral Nutrition, BNST (British Nutrition Screening Tool), CNST (Canadian Nutrition Screening Tool), CONUT (Controlling Nutritional Status), EDC (ESPEN Diagnostic Criteria for Malnutrition), GNRI (Geriatric Nutrition Risk Index), INSYST (Imperial Nutritional Screening System), MST (Malnutrition Screening Tool), MSTC (Malnutrition Screening Tool for Cancer), NRI (Nutritional Risk Index), NRS-2002 (Nutritional Risk Screening), NUFFE (Nutritional Form For the Elderly), SGA (Subjective Global Assessment), SNAQ (Short Nutritional Assessment Questionnaire), Spinal NST (Spinal Nutritional Screening Tool) R-NST (Renal Nutritional Screening Tool), 3-MinNS (3 Minute Nutrition Screening)

3.4.2 Tools with evidence of validity, reliability and acceptability

The available validity, reliability and acceptability data are summarised in tables 6 and 8. Table 7 assesses how well each tool encompasses the criteria in the chosen definitions. Assessment of bias is shown in table 9.

3.4.3 Sarcopenia

In total two tools were found that were validated for the assessment of sarcopenia (SPSM and SARC-F). Three other tools assessed muscle function but no other tools made an assessment of muscle strength, mass or wasting. Both tools which were validated for the assessment of sarcopenia were done so in the community setting. They agreed with the SCWD diagnostic criteria but the SARC-F showed variation in agreement against the three consensus definitions it was validated against (EWGSOP, IWGS criteria and the Asian working group for sarcopenia). The SARC-F had good specificity (94.2-99.1%) but poor sensitivity (3.8-9.9% dependent on sex) and also showed good agreement (0.78-0.90). Values for the SPSM were not assessed.

3.4.4 Cachexia

Only one tool had been validated for the screening of cachexia – the CASCO. Overall six tools quantified weight loss within a specified time frame, with a further three quantifying it within an unspecified time frame. Sixteen tools characterised weight loss as unintentional. Only seven tools asked about the presence of underlying disease and only the CASCO took into account the

presence of raised inflammatory markers and quality of life. Sensitivities and specificities were not recorded for the CASCO but it scored well in the assessment of its validity with it being able to quantitatively classify stages of cachexia. Its ability to predict patient's outcome was not assessed.

3.4.5 Malnutrition

Nineteen screening tools were found to be validated for the assessment of malnutrition. However, only twelve of these incorporated a question about dietary intake or decline. Six measured percentage weight loss over time and 13 assessed BMI. In particular those tools which had high sensitivities and specificities (MSTC and SNST) did not encompass all parts of the agreed definition: The SNST did not assess BMI and the MSTC made no assessment of quantifying weight loss within a specified time frame. The 3 MinNS was the tool which incorporated the consensus definition criteria and also had high sensitivities and specificities (>80%).

Table 6 Psychometric evaluation of tools to measure unintentional weight loss

◆ = tool assessed for and found to be valid X = tool assessed for and found not to be valid - = tool not assessed for/not enough information provided

	Scale	Environment	Context (OP/IP)	Face validity	Content validity	Factor analysis	Construct validity	Discriminant validity	Predictive validity	Test-retest	Internal consistency	Responsiveness	Acceptability	Time to complete
Sarcopenia	SARC-F	Community dwelling	Outpatients	◆	-	-	◆	-	◆	-	◆	-	◆	-
	SPSM	Community dwelling	Outpatients	-	-	◆	X	◆	◆	◆	◆	◆	-	◆
Cachexia	CASCO	Oncology	Outpatients	◆	◆	◆	◆	◆	-	-	◆	◆	-	-
Malnutrition	BAPEN	Acute medical and elderly care	Inpatients	◆	-	-	◆	-	-	◆	-	◆	◆	◆
	BNST	Spinal cord injuries	Inpatients	◆	-	◆	-	-	-	◆	-	-	-	-
	CNST	Medical and surgical	Inpatients	◆	-	-	◆	-	◆	◆	-	-	-	-
	CONUT	Medical and surgical	Inpatients	◆	-	X	◆	-	-	-	-	◆	◆	-
	EDC	Medical and surgical	Inpatients	◆	-	-	-	-	◆	-	◆	-	-	-
	GNRI	Acute geriatrics	Outpatients	◆	-	◆	-	-	◆	-	-	-	◆	-
	INSYST	Medical, surgical and oncology	Inpatients	◆	◆	-	◆	-	-	◆	-	◆	◆	◆
	MST	Medical, surgical and oncology	Inpatients Outpatients	◆	◆	◆	◆	-	◆	◆	◆	-	◆	-
	MSTC	Oncology	Inpatients	◆	X	◆	◆	-	-	-	-	-	X	◆

	MUST	Medical, surgical and oncology	Inpatients Outpatients	◆	◆	X	◆	-	◆	-	-	◆	◆	◆
	NRI	Peritoneal dialysis and colorectal cancer	Inpatients	-	-	◆	-	-	◆	-	-	-	◆	-
	NRS-2002	Elderly, medical and surgical	Inpatients	◆	◆	-	◆	-	◆	◆	-	◆	◆	◆
	NUFFE	Elderly care rehab	Inpatients Outpatients	◆	◆	◆	◆	-	◆	◆	◆	-	-	-
	R-NST	Renal	Inpatients	◆	◆	◆	◆	-	-	-	-	-	X	◆
	SGA	Elderly, renal, medical and surgical	Inpatients	◆	◆	◆	◆	-	◆	◆	◆	-	-	-
	SNAQ	Medical, surgical and oncology	Inpatients Outpatients	◆	◆	-	◆	◆	◆	◆	-	◆	◆	◆
	SNST	Medical and surgical	Inpatients	◆	-	◆	-	◆	◆	◆	◆	◆	◆	◆
	Spinal NST	Spinal cord injuries	Inpatients	◆	-	◆	-	-	-	◆	-	-	◆	◆
	3-MinNS	Medical and surgical	Inpatients	◆	◆	-	◆	◆	◆	◆	-	◆	◆	◆

Table 7 Domains assessed by tools to measure relevant parameters required to identify risks of malnutrition, sarcopenia and cachexia

		Patient reported weight loss						BMI & FFM measurements		Nutritional intake				Assessment of muscle mass and function		Disease state		Measures of metabolic derangement				Quality of life
Disease	Screening tool	Weight loss quantified within specified time frame	Weight loss quantified without timeframe	Weight loss unquantified with time frame	Weight loss unquantified, without time frame	UWL specified	Muscle mass	BMI	FFMI	Loss of appetite	Poor dietary intake/ intake decline	Supplemental feeding in use?	Symptoms that would prevent eating e.g. vomiting, ulcers	Physical performance	Muscle strength	Presence of illness	Fatigue	Increased inflammatory markers	Anaemia	Low serum albumin	Other blood Tests e.g. glucose/urea	QOL
Sarcopenia	SARC-F	X	X	X	X	X	X	X	X	X	X	X	X	✓	✓	X	X	X	X	X	X	X
	SPSM	X	X	X	X	X	X	✓	✓	X	X	X	X	✓	✓	X	X	X	X	X	X	X
Cachexia	CASCO	X	✓	X	X	✓	X	X	✓	✓	✓	X	X	✓	X	X	✓	✓	✓	✓	✓	✓
Malnutrition	BAPEN	X	X	X	✓	✓	X	✓	X	X	✓	X	X	X	X	X	X	X	X	X	X	X
	BNST	X	X	X	✓	✓	X	✓	X	X	✓	X	X	X	X	X	X	X	X	X	X	X
	CNST	X	X	✓	X	✓	X	X	X	X	✓	X	X	X	X	X	X	X	X	X	X	X
	CONUT	X	X	X	X	X	X	✓	✓	X	X	X	X	X	X	X	✓	X	X	✓	✓	X
	EDC	X	X	✓	✓	X	X	✓	X	X	X	X	X	X	X	X	X	X	X	✓	X	X
	GNRI	X	X	✓	X	✓	X	X	X	X	✓	X	X	X	X	X	✓	X	X	X	X	X
	INSYST	✓	X	X	X	✓	X	X	X	✓	X	X	X	X	X	X	X	X	X	X	X	X
	MST	X	✓	X	X	✓	X	✓	X	X	✓	X	X	X	X	X	✓	X	X	X	X	X
	MSTC	X	X	X	X	✓	X	✓	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	MUST	✓	X	✓	X	✓	X	X	X	X	X	✓	X	X	X	X	✓	X	X	X	X	X
	NRI	X	X	X	X	x	X	✓	X	X	✓	X	X	X	X	X	X	X	X	X	X	X
	NRS-2002	X	✓	X	X	✓	X	X	X	X	✓	✓	X	X	X	X	✓	X	X	X	X	X
	NUFFE	X	X	X	✓	X	X	✓	X	X	✓	✓	X	X	X	X	X	X	X	X	X	X
	R-NST	✓	X	X	X	✓	X	X	X	✓	✓	✓	X	X	X	X	✓	X	X	X	X	X
	SGA	✓	X	X	X	✓	X	✓	X	✓	✓	✓	X	X	X	X	X	X	X	X	X	X
	SNAQ	X	X	X	✓	✓	X	X	X	X	✓	✓	X	X	X	X	X	X	X	X	X	X
	SNST	X	X	X	X	✓	X	✓	X	✓	✓	✓	X	X	X	X	✓	X	X	X	X	X
	SpinalNST	✓	X	X	X	✓	X	✓	X	X	✓	✓	X	X	X	X	✓	X	X	X	X	X
	3-MinNS						X													✓		

Table 8 Sensitivity, specificity, predictive values, and reproducibility of the studies included

Author	Screening tool	Sensitivity	Specificity	PPV	NPV	Agreement
Woo et al, 2014	SARC-F	3.8-9.9	94.2-99.1	8.4-54.8	78.4-94.9	0.78-0.90
Miller et al, 2009	SPSM	-	-	-	-	-
Argiles et al, 2017	CASCO	-	-	-	-	-
Weekes et al, 2004	BAPEN	-	-	-	-	0.77
Mirmiram et al, 2011	BNST	86.7	61.7	79.1	73.1	0.74
Laporte et al, 2015	CNST	72.6	85.1	81.2	77.0	0.88
Ignacio et al, 2005	CONUT	92.3	85	-	-	0.488
Guerra et al, 2017	EDC	17.1	98.3	89.1	58.9	0.803
Abd-El-Gawad et al, 2014	GNRI	83.1	51.2	78.95	58.33	0.713
Tammam et al, 2009	INSYST	95-100	65-83	-	-	0.73
Kim et al, 2011	MST	93	93	98.4	72.7	0.7
Ferguson et al, 1999		100	92	80	100	0.83
Isenring et al, 2006		67	86	-	-	0.53
Neelemaat et al, 2011		49	86	-	-	0.33
Nursal et al, 2005		73	55	-	-	0.28
Young et al, 2013		73	70	-	-	-
Wu et al, 2012		39	93	-	-	0.21
Bhuachalla et al, 2018		39.4-100	47-74.6	-	-	0.71
Leipold et al, 2018		72.2	83.8	69.6	85.4	-
Kim et al, 2011	MSTC	94	84.2	67.8	97.6	0.70
Boleotome et al, 2012	MUST	80	89	100	100	-
Leistra et al, 2013		75	94	43	98	-
Sharma et al, 2017		69.7	75.8	75.4	70.1	0.49
Neelemaat et al, 2011		96	80	-	-	-
Kyle et al, 2006		61	79	-	-	-
Young et al, 2013		87	86	-	-	-
Almedia et al, 2012		85	93	-	-	-
Velasco et al, 2011		72	90	-	-	-
Bhuachalla et al, 2018		20.8-72.8	48-98.3	-	-	0.816
Prasad et al, 2012	NRI	92.9	32.39	80.41	60.53	0.63
Faramarzi et al, 2013		66	60	64	62	0.267
Bhuachalla et al, 2018		21.2-95	21.2-92.1	-	-	-
Neelemaat et al, 2011	NRS-2002	92	85	-	-	-
Kyle et al, 2006			93	-	-	-
Young et al, 2013		62	83	-	-	-
		90	89	-	-	-

Almeida et al, 2012		80	85	-	-	-
Bauer et al, 2005			87	-	-	-
Velasco et al, 2011		70				
Soederhamn et al, 2002	NUFFE	74				
Xia et al, 2016	R-NST	71	86	-	-	-
Duerksen et al, 2000	SGA	97.3	74.4	88.0	93.6	0.95
Cooper et al, 2002		-	-	-	-	-
Moriana et al, 2014		59-68	61-65	41-42	70-83	0.6
Kruizenga et al, 2005	SNAQ	-	-	-	-	-
Leistra et al, 2013		79	83	70	89	-
Harada et al, 2017		43	99	78	96	-
Neelemaat et al, 2011		43	99	-	-	-
Young et al, 2013		75	84	-	-	-
Susetyowati et al, 2014	SNST	79	90	-	-	-
Wong et al, 2011	Spinal NST	97	80	78	92	0.57
Lim et al, 2009	3-MinNS	85.7	76.1	62	92	-
		86	83	67	94	-

Table 9 Newcastle-Ottawa scale adapted for cross-sectional studies

	Woo et al, 2014	Miller et al, 2009	Argiles et al, 2017	Weekes et al, 2004	Mirmiram et al, 2011	Laporte et al, 2015	Ignacio et al, 2005	Guerra et al, 2017	Abd-El-Gawad et al, 2014	Tammam et al, 2009	Ferguson et al, 1999	Isenring et al, 2006	Neelemaat etal, 2011	Nursal et al, 2005	Young et al, 2013	Bhuachalla et al, 2018	Leiopold et al, 2018	Wu et al, 2012	Kim et al, 2011	Boleo-Tome et al, 2012	Leistra et al, 2013	Sharma et al, 2017	Kyle et al, 2006	Almedia et al, 2012	Velasco et al, 2011	Prasad et al, 2012	Faramarzi et al, 2013	Bauer et al, 2005	Soederhamn et al, 2002	Duerksen et al, 2000	Cooper et al, 2002	Moriana et al, 2014	Kruizenga et al, 2005	Harada et al, 2017	Susetyowati et al, 2014	Wong et al, 2011	Xia et al, 2016	Lim et al, 2009
Selection (max 5 stars)																																					
1.Representativeness of the sample	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
2. Sample size justified	*	*	*	*	*	*			*			*	*	*	*	*	*	*			*		*	*	*	*		*	*		*	*	*	*	*	*	*
3. Response rate satisfactory			*												*	*	*																					
4. Ascertainment of exposure (validated measurement tool used)	*	*	*	*	*	*	**	**	*	**	**	**	*	**		*		**	**	**	*	**	**	**	**	**	**	**	**	*	**	**	*	*	**	*	*	**
Comparability (max 2 stars)																																					
1.The study controls for disease severity	*	*	*					*	*														*							*							
2. Study controls for other confounding factors	*	*							*										*				*										*				
Outcome (max 3 stars)																																					
1. Assessment of outcome	*				**	**	**			**	**	*				**		**				**								**	**						
2. Appropriate statistical test described	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Total (Out of 10)	7	6	5	5	6	6	6	5	6	6	6	6	4	5	4	7	4	7	5	4	4	6	6	5	6	5	4	5	5	7	7	6	4	6	6	4	5	5

*= Assessed in study and found to be present. Score <5 = high risk of bias

3.5 Discussion

3.5.1 Overview

Whilst recent systematic reviews have described the results of studies examining malnutrition screening tools, to our knowledge this is the first review to examine tools that have been validated against another to assess cachexia, sarcopenia and malnutrition. There has only been one prior review on tools for cachexia, sarcopenia and malnutrition (287). This review did not include psychometric evaluation, did not comment on the validity of the tools, nor compare them to the agreed consensus definitions. Existing systematic reviews of malnutrition screening tools have been limited to describing tools that are non-disease specific and 'quick and easy' or have been narrative in nature.

Thirty-eight studies describing 22 tools were identified and judged for validity against a reference method. In the absence of a generally recognised gold standard for screening, assessment by a professional, DXA, CT, MRI, anthropometry or the screening tools SGA and MNA were considered 'valid' reference methods by our research group (247,278–280). Although cross sectional imaging is now used routinely for body composition analysis, only three tools identified were validated against CT. The heterogeneity in populations, age groups, tools and reference methods was large, and therefore pooling of results was impossible. Most tools had only been tested in one population making the drawing of any definitive conclusions difficult.

There were too few disease-specific tools to conclude which would be superior for different disease processes.

3.5.2 Problems with current screening tools

For the generalised adult population, all tools showed inconsistent results regarding their validity. The SGA which is often considered to be the industry standard (288) and against which many tools are validated has not itself been well validated. It performed well against the diagnostic criteria but sensitivities and specificities were either not recorded or poor. Arguably the most well-known tools 'MUST' and 'NRS-2002' showed a variation in results from poor to good (261,263,268–273,276), and consistency between groups in which the tools were studied was poor. The less well-known NUFFE showed good validity, but it has been described in only a small volume of literature and is not implemented widely (277). The "quick and easy" screening tools, including SNAQ and MST performed reasonably (sensitivities ~80%) in most studies in which they were used (259–264,269,281,282). Of note because these tools are quick, they require a further detailed assessment by a qualified health professional if screening is positive. They also miss approximately 20% of at risk patients at initial screen and therefore may be more useful in screening high risk patients.

The tool which performed the best for malnutrition was the 3MinNS (286). It showed high sensitivity and specificity (>80%) and accurately encompassed

the correct diagnostic criteria (percentage weight loss over specified time and measurement of BMI) for malnutrition. It was validated in acute medical and surgical patients and proved quick and easy to complete. It has only been validated in one paper and therefore it cannot be assumed that it would perform as well in different patient populations. Both tools which assessed sarcopenia (SPSM, SARC-F) scored well against the agreed definition (249,250). However, the SPSM required transport of equipment and the SARC-F had a very low sensitivity (247,249). The CASCO was the only validated screening tool for cachexia (251). It performed well against diagnostic criteria, but sensitivities and specificities were not recorded. It has also only been validated in the cancer setting; more work would be needed to validate the tool in other cachectic populations or the general adult population.

Most tools were validated in the adult hospital inpatient setting. Tools for sarcopenia have only been validated in healthy, community dwelling individuals (249,250). Length of hospital stay is diminishing worldwide and outpatient nutritional screening is advocated to pick up patients at risk. In this review, we identified eight studies in which outpatients were included. More studies focussing on the construct and predictive validity of tools for outpatient screening are warranted, especially since care is shifting to this setting.

The tool which appeared to have the broadest coverage was the CASCO (251). It is the only tool which screens for cachexia, but also picks up many of

the variables required for a diagnosis of malnutrition. However, assessment of muscle mass or function (required for sarcopenia) is not included. One previous review showed that 20 screening tools appeared relevant for starvation, but none contained all the currently accepted components needed to screen for sarcopenia and cachexia risk (287). This study supports this.

3.5.3 Outlook and recommendations for future tools

A screening tool needs to be developed that encompasses the criteria to pick up all three possible syndromes. This concept is supported by the notion that, in the human being, there may be no “pure” phenotype of cachexia, as it is usually associated with reduced food intake (potential for malnutrition) and increasing age (increasing sarcopenia) (289). There is also currently a lack of agreement as to the diagnostic criteria of each syndrome and the relative importance of body composition analysis and the nature of depleted tissue within each definition. It was hypothesised that the overlap between syndromes could be illustrated as in figure 20 along with the identified best performing tools for each aspect.

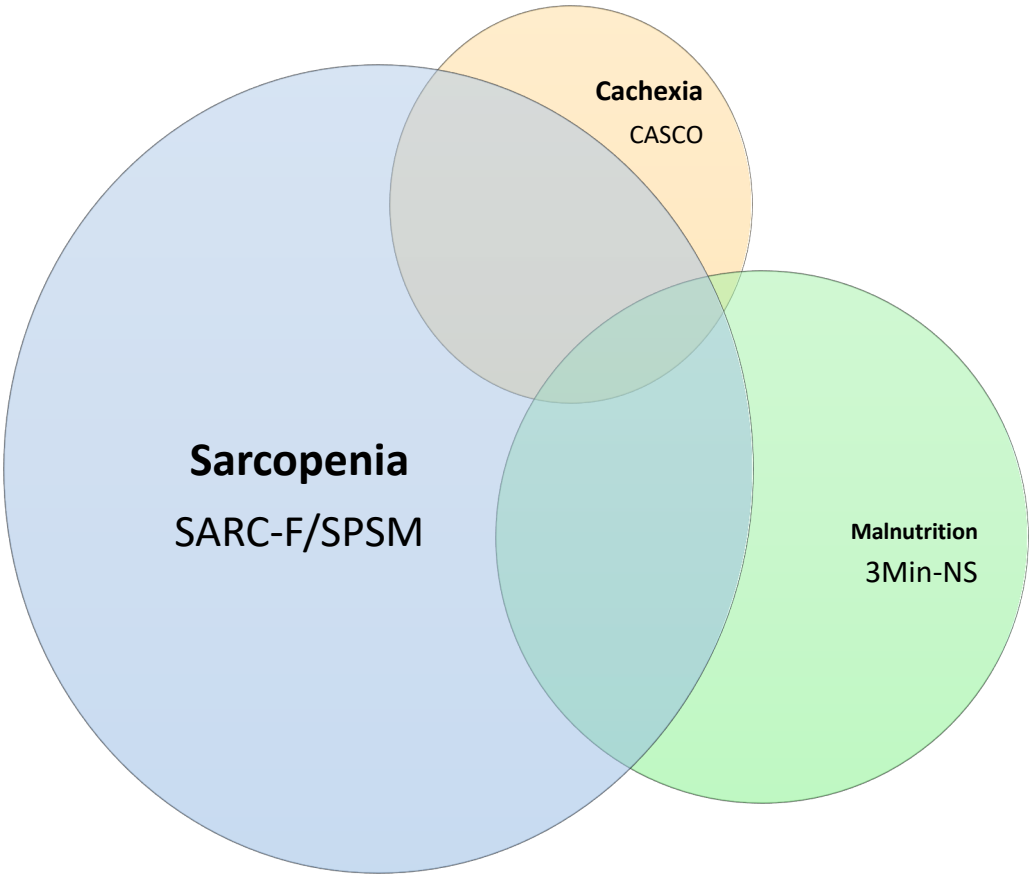


Figure 20 Overlap between cachexia, sarcopenia and malnutrition

Figure demonstrates similar clinical presentation between the three conditions

There are clearly many existing validated screening tools (at least for malnutrition). It is unlikely that any further novel tools will be devised without breakthroughs in biomarker development. We therefore suggest that the ideal composite tool incorporate a stepwise assessment of nutritional status; oral intake; disease status; patient age; muscle mass/function; and metabolic

derangement. The presence of underlying disease is a key question in order to stratify the syndromes. Suggested components for use in creating a new tool are depicted in **table 10**.

1. Quantification of weight loss
2. Measurement of BMI
3. Assessment of appetite/dietary intake and decline
4. Underlying health state – is there the presence of chronic disease?
5. Take into account patient's age (i.e. >60 more likely to be sarcopenic)
6. Assessment of muscle mass and function
7. Measurement of metabolic derangement/raised CRP
8. Gender

Table 10 Suggested components for use in creating a new screening tool

By screening for all three syndromes, it will allow for a more targeted intervention. Screening for cachexia, sarcopenia or malnutrition is not warranted unless it is accompanied by an intervening care plan. It would be expected that an adequate intervention would prevent any further decline in health status and therefore lead to a positive effect on disease outcome.

Most studies did not comment on intervention, which depending on the balance of the three syndromes may need to include varying attention to nutrition, exercise and measures to combat inflammation.

3.5.4 Strengths, limitations and assessment of bias

One of the strengths of this review is that it provides a complete overview of tools that have been validated for cachexia, sarcopenia and malnutrition. It did not describe reliability, repeatability or other clinical outcome measures in any great detail. The review used the consensus definitions of each syndrome, however, many other definitions exist. There were a number of study limitations. There was a risk of bias when assessing each tool for their predictive validity. Studies may have been biased if they did not adjust for factors such as cancer stage or disease severity. As clinical outcome is affected by more than just nutritional status alone adjusting for these variables is important. Nutritional intervention is likely to improve outcomes for malnutrition but potentially not for age-related sarcopenia or established cachexia. Only one study discussed whether they did this. There is no agreed 'gold standard' tool and therefore cross sectional imaging and the SGA and MNA were chosen based on the results of previous studies (247). Tools that were compared to potentially less valid standards were also included to allow a wider analysis. Full nutrition assessments were different in each study ranging from anthropometric to biochemical measures and full assessment by a medical professional. Conclusions from this study were based upon the

original papers in which there may have been varying definitions with regards to the subject group, syndrome or assessment undertaken. Another potential limitation is that modified versions of pre-existing tools were excluded. They were excluded as reliability and validity data would only relate to the modified tool and it was therefore difficult to assess improvements from the original. It is possible that these tools were being improved or evaluated more thoroughly.

3.6 Conclusion

This study has highlighted that many practitioners who regularly come into contact with patients suffering from weight loss are not able to easily screen between the conditions of cachexia, sarcopenia and malnutrition as there is no one validated tool which can be implemented for the assessment of all three conditions. The adaptation of existing screening tools incorporating all relevant criteria described in this review would be optimal for diagnosis and to direct the content of complex interventions.

CHAPTER 4

AGE AND SEX RELATED VARIATIONS IN CT DERIVED BODY COMPOSITION FOR OESOPHAGEAL CANCER

4.1 Overview of chapter

The previous chapter has highlighted the difficulties in screening patients for cachexia. This chapter investigates the use of the current gold standard for the assessment of muscle mass in cancer patients. CT based cutoff values for determining low skeletal muscle mass are sex and BMI-specific, and have historically been driven by optimal stratification to predict mortality in cancer patients. Elderly patients may be sarcopenic at diagnosis and more patients are obese making it increasingly difficult to detect cancer associated muscle wasting.

4.1.2 Objectives

The aim of the forth chapter was to investigate the age and sex-specific differences in skeletal muscle, visceral (VAT) and subcutaneous adipose tissue (SAT) in patients suitable for resection of oesophageal cancer.

4.1.3 Methods

This was a retrospective, observational, single-centre study. Medical records of patients who underwent neoadjuvant chemotherapy (NAC) and or oesophagectomy were reviewed. CSA at the third lumbar vertebrae on routine staging and post-chemotherapy CT were measured, and skeletal muscle, visceral and subcutaneous adipose tissue indexed for height. Intra-muscular fat CSA was also recorded. Weight data was not available at the time of each

CT. The association between age, sex, skeletal muscle and adipose measurements were examined.

4.1.4 Results

108 patients were included, 71 of those had undergone NAC. Failure to complete NAC occurred in 11 patients. Significant differences in body composition were seen between males and females and those above and below 60 years of age. Males had a higher baseline muscle and visceral adipose tissue mass ($p=0.0123$ and $p=0.016$) whereas females had higher levels of subcutaneous fat ($p=0.017$). Older patients and females lost significantly more fat following NAC ($p=0.004$ and $p=0.008$ respectively). Patients of all ages were found to lose muscle mass following NAC but there was no difference between rates of wasting by age or sex.

4.1.5 Conclusion

This is the first study to report variation in body composition in oesophageal cancer by age and sex. Rates of adipose wasting appear to be clinically significant in oesophageal cancer. Further studies are needed to define alterations in different adipose depots during cancer progression and their prognostic value.

4.2 Introduction

Several studies have used CT to evaluate outcomes in relation to low muscle mass in various groups e.g. cancer patients, patients undergoing surgery and those in intensive care. Most studies associate lower skeletal muscle mass with poor oncological and surgical outcomes. The majority of these studies demonstrate a linear relationship between skeletal muscle index and outcome, and very few have created cutoff points in order to distinguish between “low” and “normal” skeletal muscle mass. It is becoming increasingly apparent that cut points that were set for use in cancer patients cannot be applied to other groups and to older patients who may be sarcopenic by definition at diagnosis. Interpretation of muscle parameters is also made difficult since reference values in a healthy population are lacking.

The body composition of contemporary cancer patients is highly variable with respect to the features of muscle and fat mass as well as the distribution of fat in abdominal and subcutaneous regions. The WHO identifies more than 600 million adults as obese (BMI >30 kg/m^2) meaning that BMI no longer provides an adequate assessment of body composition (210).

Low subcutaneous adipose tissue index (SATI) has been independently associated with increased mortality in gastrointestinal and respiratory cancer patients. In the presence of sarcopenia however, the longest survival was observed in patients with high subcutaneous adiposity (223). Others have

highlighted sarcopenic obese patients to have particularly bad outcomes. The concept and impact of sarcopenic obesity therefore requires further investigation.

Patients do not also necessarily lose or gain muscle and fat in equal measures. Muscle and adipose tissue parameters are likely to be associated with age, sex, BMI and ethnicity and these characteristics should be taken into account when interpreting body composition. In order to better quantify the severity of muscle and adipose tissue loss, in the elderly in particular requires scoring systems or reproducible cutoff values for differing age groups. With this in mind this chapter aims to investigate the age and sex related differences in body composition in patients suitable for potentially curative resection of oesophageal cancer.

4.3 Methods

This was a retrospective, observational single centre study. All data was acquired as part of routine clinical practice.

4.3.1 Study population

Medical records of individuals undergoing potentially curative resection for oesophageal malignancy were retrieved. These were all patients who had been recruited to previous cachexia studies and had undergone rectus muscle biopsy. In order to be included patients had to have an available pre-operative

staging CT. Patients with locally advanced disease who had a WHO performance status 0-2 had preoperative chemotherapy [Epirubicin, Cisplatin and Capecitabine (ECX) or Epirubicin, Cisplatin and Fluorouracil (ECF) or Cisplatin and Fluorouracil (Cis5FU)]. For those patients who underwent pre-operative chemotherapy a post-chemotherapy staging scan was also required.

4.3.2 CT scan analysis

CT analysis was performed as previously described. Cachexia was defined as >2% weight loss over the preceeding six months and low muscularity on CT.

Due to variation in the interval between CT images acquired before and after chemotherapy (88 ± 31 days), change (Δ) in parameters of body composition that were observed between the diagnostic and post-chemotherapy CT assessment are expressed as Δ/100days.

To determine the rate of either skeletal muscle or total adipose tissue loss during chemotherapy, patients were divided into four groups: (i) stable (gain or no loss); (ii) minimal rate of loss (iii) moderate rate of loss, or: (iv) severe rate of loss. Minimal rate of loss in muscle mass was defined by a loss of no greater than $6cm^2$ Δ/100days of CSA. Moderate rate of loss was defined as loss of between $-6cm^2$ to $-18cm^2$ Δ/100days, whilst severe rate of loss was considered in skeletal muscle CSA decreased by more than $18cm^2$ Δ/100days. In comparison, minimal rate of loss in total adipose tissue was defined by a loss

of no greater than 16cm^2 Δ/100days of CSA. Moderate rate of loss was defined as loss of between -16cm^2 to -70cm^2 Δ/100days, whilst severe rate of loss was considered in total adipose tissue CSA decreased by more than 70cm^2 Δ/100days (290).

4.3.3 Outcome measures

The primary outcome measure of this study was variation in parameters of body composition (intra-muscular fat, skeletal muscle, visceral and subcutaneous adipose cross-sectional areas, SMI, SATI and VATI) by age and sex. Secondary outcomes were change in body composition following neoadjuvant chemotherapy (NAC).

4.3.4 Statistical analysis

Data were exported to Microsoft Excel (Microsoft Office, Washington, USA). Statistical analysis was performed using SPSS (version 24.0, IBM Corp., Armonk, USA). Patients were analysed based on age and sex. For staging scan data all patients were analysed by decade of life. For patients who underwent chemotherapy patients were divided into two age groups (<60 years or 60 years and over). Dependent on their distribution, continuous variables are presented as either mean (standard deviation) or median (interquartile range, IQR). Comparison of continuous variables was performed using the Independent-Samples T test or Mann-Whitney U test respectively. Comparison of continuous variables across more than two independent

groups was performed by one-way analysis of variance or the Kruskal-Wallis test. Categorical variables were compared using the chi-squared or Fisher's exact tests. P values <0.05 were considered statistically significant.

4.4 Results

4.4.1 Patient characteristics

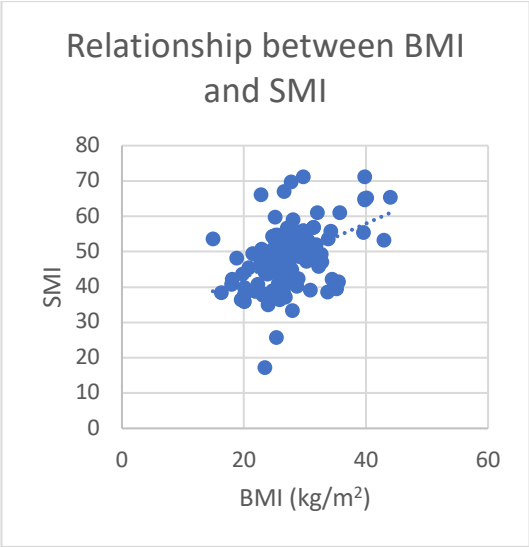
One hundred and eight patients (84 males and 24 females) were included. Baseline patient characteristics are presented in table 11. Patients were predominantly diagnosed with adenocarcinoma (81.65%) of the lower oesophagus or gastroesophageal junction. As all patients were deemed suitable for potentially curative surgery; none had metastatic disease. There were no differences between weight, BMI and percentage weight loss at diagnosis between age groups. Staging BMI, a marker of nutritional status often used in the outpatient setting was found to correlate poorly with muscle and adipose indices (figure 21).

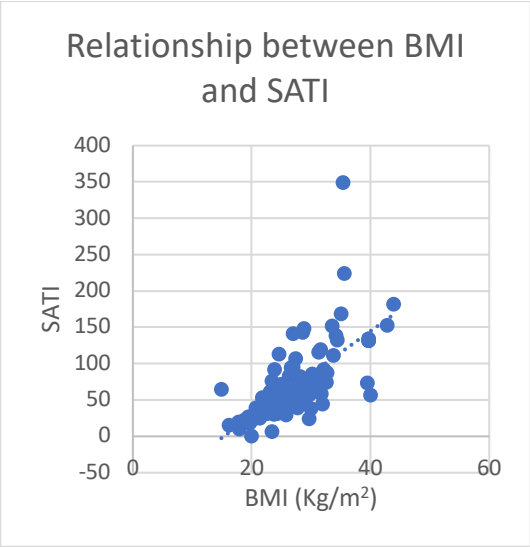
	Age group (years)					
	<49	**50-59**	**60-69**	**70-79**	**80-89**	**P Value**
N	5	26	38	34	5	N/A
Male:Female	4:1	19:7	25:13	31:2	5:0	N/A
Staging weight (kg)	80.04 (27.33)	78.76 (11.08)	84.38 (20.98)	81.32 (13.92)	69.06 (15.83)	0.523
% weight loss at diagnosis	6.36 (6.57)	4.87 (5.92)	4.63 (5.56)	3.48 (4.44)	2.11 (2.66)	0.523
BMI (Kg/m^2)	26.76 (7.92)	26.94 (4.12)	28.85 (6.79)	26.30 (4.23)	22.82 (4.27)	0.243
Type of cancer (ACC:SCC:Other)	5:0:0	21:4:1	31:7:0	27:6:1	5:0:0	N/A
Cancer location (UO:LO:GOJ)	0:4:1	2:17:7	2:29:7	1:27:6	0:4:1	N/A

Table 11 Staging patient demographic details

Data are Mean (SD)

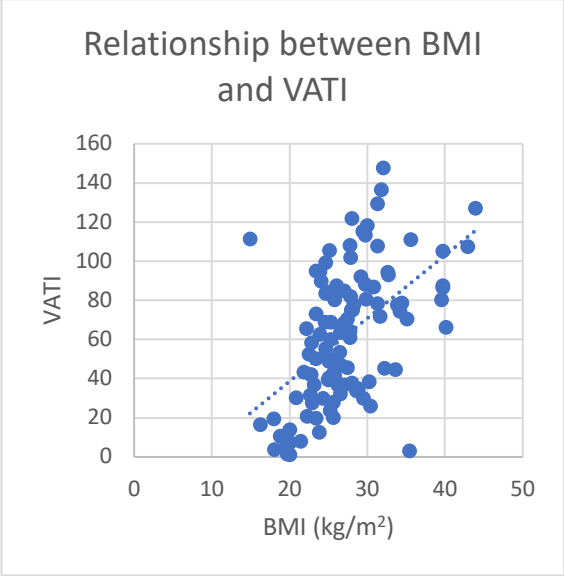
ACC – adenocarcinoma, SCC – squamous cell carcinoma, UO – upper oesophagus, LO – lower oesophagus, GOJ – gastroesophageal junction





R^2=0.2072

R^2 = 0.4437



R^2 = 0.2699

Figure 21 Variation in muscularity and adiposity on staging CT scan in patients suitable for potentially curative resection of oesophageal cancer (N=108).

BMI and SMI/SATI/VATI are poorly correlated.

4.4.2 Variation in staging body composition parameters

None of the patients under 50 years were sarcopenic at diagnosis, this increased to 80% in the >80 years group with those in the middle displaying a similar prevalence (42, 40 and 58% for the age 50-59, 60-69 and 70-79 year groups respectively). Although not significant staging SMI was the lowest in the 80-89 years age group [43.13 (3.54) compared with 55.29 cm^2/m^2 (10.52) in the <50 group, p=0.207]. The oldest patients also had the lowest VATI and SATI on staging scan [30.50 (12.85) p=0.005 and 35.92 cm^2/m^2 (15.39) p=0.132 respectively]. IMAT CSA was significantly different between age groups (p=0.003). Staging TATI and total adipose CSA also differed by age (p=0.030 and p=0.016). This is summarised in table 12 and figure 22.

Again none of the patients under 50 met the diagnostic criteria for cachexia (>2% weight loss and low muscularity on CT), but this increased to 40% in those over 80 years. Staging SMI and muscle CSA was lower in cachectic patients but there were no differences in any measures of adipose tissue at diagnosis between those who were cachectic and non-cachectic by consensus definition (2% weight loss and low muscularity). Sarcopenic obesity was apparent in 10 out of the 108 patients.

	Age group (years)					
	<49	**50-59**	**60-69**	**70-79**	**80-89**	**P Value**
Staging SMI	55.29 (10.52)	49.34 (9.29)	49.19 (10.23)	45.84 (7.91)	43.14 (3.54)	0.207
Staging SATI	63.34 (46.40)	68.25 (40.38)	87.89 (66.53)	56.78 (22.31)	35.92 (15.39)	0.132
Staging VATI	38.52 (41.05)	53.71 (30.51)	62.49 (34.25)	76.55 (33.74)	30.50 (12.85)	0.005*
Staging TATI	101.86 (87.25)	121.95 (57.27	150.38 (78.21)	133.33 (47.22)	66.42 (26.20)	0.030*
Staging IMAT CSA	5.51 (2.50)	9.49 (3.81)	10.28 (3.57)	15.99 (10.45)	10.27 (3.24)	0.003*
Staging Muscle CSA	165.04 (41.20)	145.31 (33.18)	144.44 (35.63)	140.79 (23.40)	130.14 (17.65)	0.569
Staging SAT CSA	186.77 (144.54)	191.18 (103.52)	257.63 (181.50)	174.49 (68.25)	108.83 (52.62)	0.102
Staging VAT CSA	117.20 (125.65)	155.56 (88.94)	186.07 (105.14)	235.49 (101.43)	93.04 (43.68)	0.003*
Total adipose CSA	309.48 (270.88)	356.23 (154.20)	453.17 (221.64)	425.48 (147.30)	212.14 (92.83)	0.016*

Table 12 Variation in staging body composition parameters by age

Mean (SD) *Denotes significant result

SMI measured in cm^2/m^2

CSA measured in cm^2

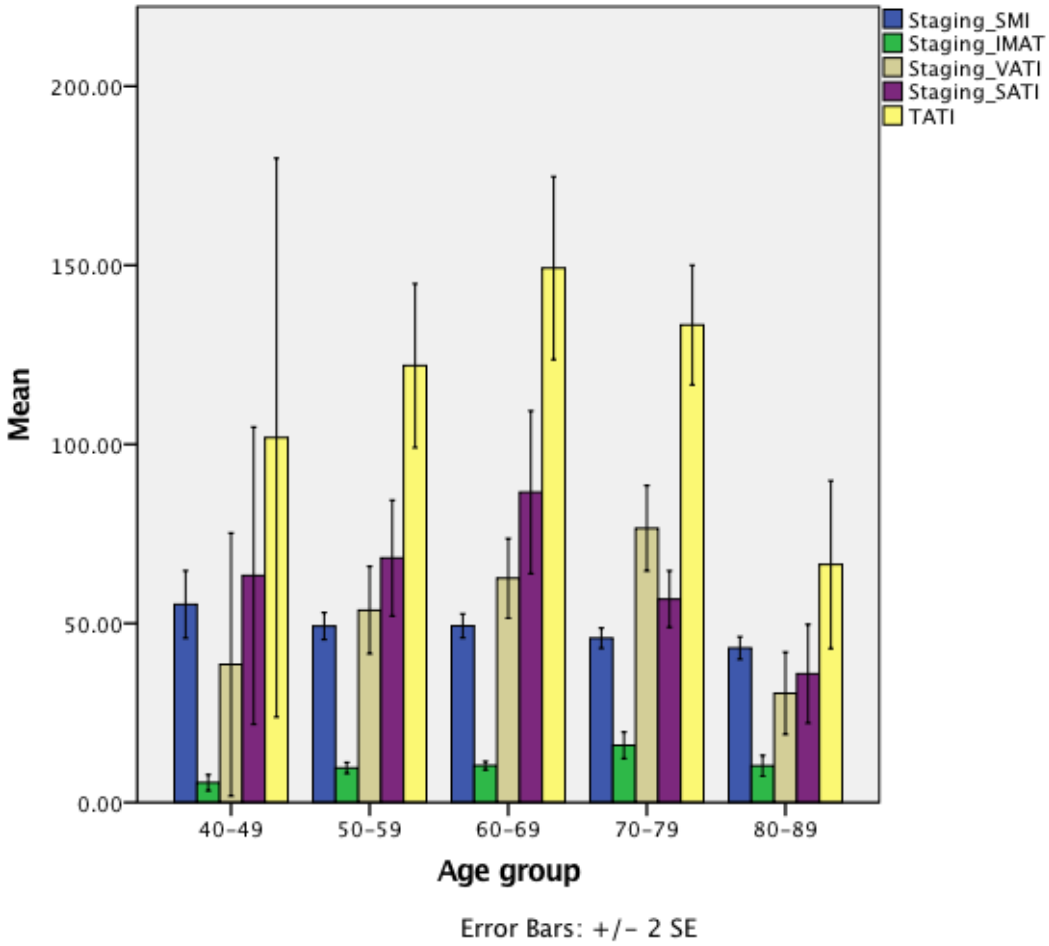


Figure 22 Staging indices on CT scan by decade of life

Staging SMI decreases with age. IMAT increases with age. Staging levels of SATI, VATI and TATI are variable by decade. SATI and TATI increases until the age of 69 then appears to decrease. Staging VATI appears to increase by decade but is lowest in those aged over 80.

SMI measured in cm^2/m^2

Women weighed significantly less at diagnosis (74 vs 83kg, p=0.029). There was no difference in BMI or weight loss at presentation between sexes (p=0.489 and p=0.658). There was a significant difference between staging muscle CSA and SMI with males displaying higher muscle mass (P<0.001, p=0.012). Females had a higher SAT CSA and SATI (290.65 vs 180.98 cm^2 and 109.11 vs 58.83 cm^2/m^2, p=0.015 and p=0.003) and males had a higher VAT CSA and VATI (204.61 vs 123.11 cm^2, p<0.001 and p=0.007). This data is summarised in table 13 and figure 23.

	Sex		P Value
	Male	**Female**	
Staging weight (kg)	83.22 (16.36)	74 (17.81)	0.029*
Staging BMI (kg/m^2)	27.00 (5.36)	27.98 (6.22)	0.489
% weight loss	4.21 (5.37)	4.83 (6.16)	0.658
Staging SMI	49.29 (9.49)	44.40 (7.61)	0.012*
Staging SATI	58.83 (31.38)	109.11 (73.90)	0.003*
Staging VATI	66.67 (34.74)	46.31 (29.81)	0.007*
Staging TATI	125.20 (56.19)	155.41 (88.30)	0.124
Staging IMAT CSA	12.15 (7.70)	9.69 (4.32)	0.055
Staging muscle CSA	151.42 (28.78)	117.52 (24.95)	<0.001*
Staging SAT CSA	180.98 (97.21)	290.65 (200.07)	0.015*
Staging VAT CSA	204.61 (104.98)	123.11 (81.06)	<0.001*
Staging total adipose CSA	397.45 (173.26)	422.64 (242.82)	0.638

Table 13 Variation in staging body composition parameters by sex

SMI measured in cm^2/m^2 CSA measured in cm^2

Mean (SD) *Denotes significant result

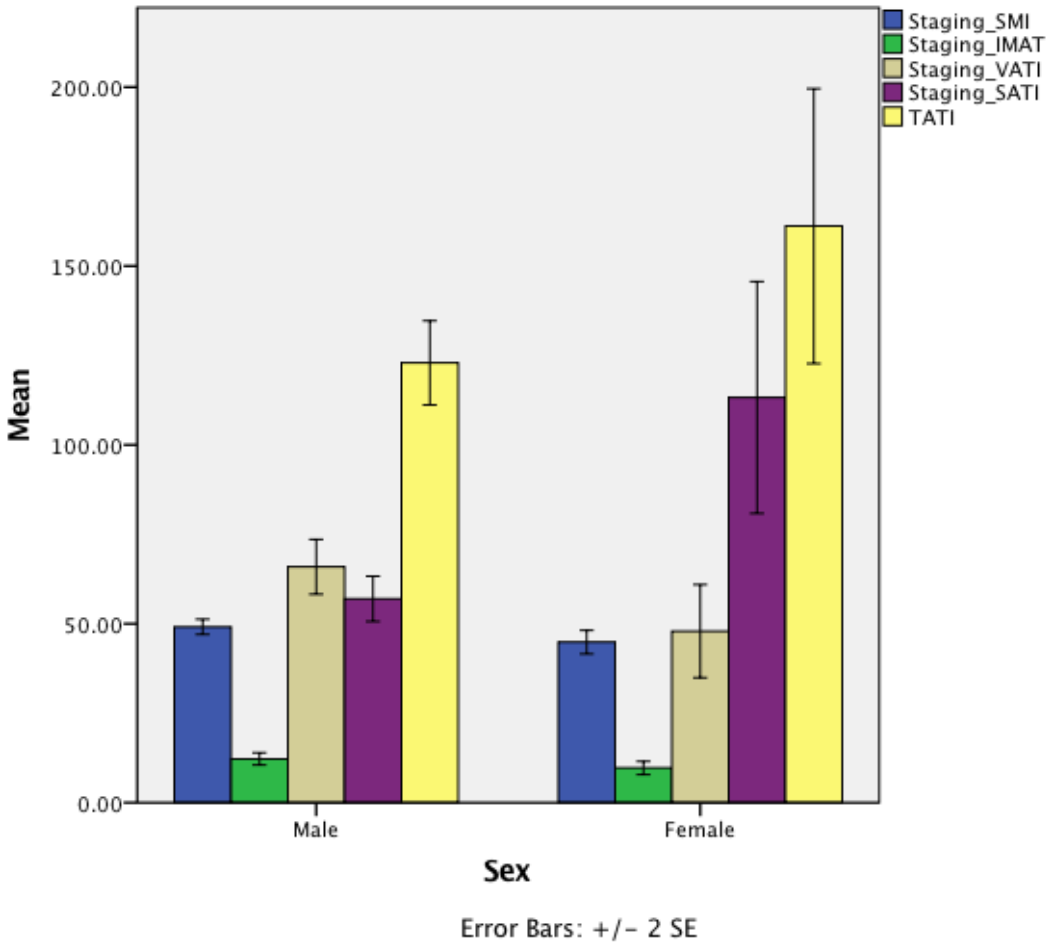


Figure 23 Staging indices on CT scan by sex

Males have higher staging SMI, IMAT and VATI whereas females have higher SATI and TATI

SMI measured in cm^2/m^2

4.4.3 Variation in body composition parameters following chemotherapy

Seventy-one of the patients included in the study had NAC and thus an available second CT. No one aged over 79 was given NAC. There were 21 patients aged under 60 years and 50 patients aged 60-79 years. There were no differences in weight, BMI or percentage weight loss at diagnosis between age groups or sex. Sarcopenia at staging was more prevalent in those aged over 60 years (42% compared with 33%). Rates of low subcutaneous fat mass were the same between groups (33% each) with those under 60 having a higher prevalence of low visceral adipose tissue mass (62% vs 30%) (figure 24). Pathology was predominantly adenocarcinoma of the lower oesophagus. This data is summarized in tables 14,15 and 16 and figure 25.

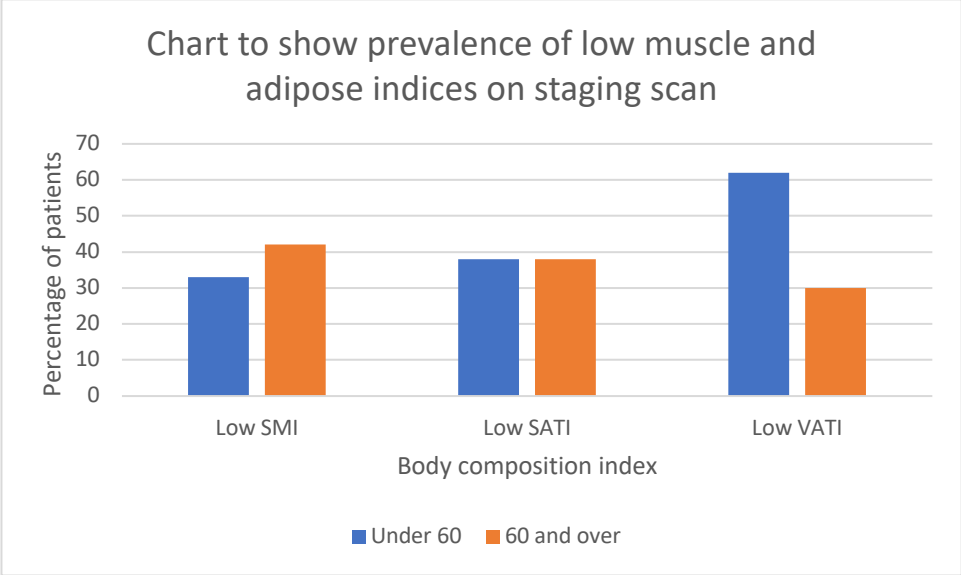


Figure 24 Prevalence of low muscle and adipose indices on staging scan in those patients undergoing neoadjuvant chemotherapy
Older patients have lower muscle and visceral adipose tissue mass

	Age group (years)		
	<60 years	**60 years and over**	**P Value**
N	21	50	N/A
Male:Female	17:4	39:11	N/A
Staging weight (kg)	79.24 (15.89)	81.63 (16.92)	0.57
Staging BMI	26.10 (5.11)	27.41 (5.79)	0.35
% weight loss	4.78 (5.35)	5.35 (6.21)	0.70
Cancer type ACC:SCC	18:3	40:10	N/A
Cancer site UO:LO:GOJ	5:10:6	1:43:6	N/A

Table 14 Demographic data for patients undergoing neoadjuvant chemotherapy

	Age (Years)		
	<60	**60 and over**	**P Value**
Staging muscle CSA	154.55 (35.71)	145.19 (28.64)	0.295
Staging SAT CSA	180.91 (117.07)	228.60 (157.44)	0.166
Staging VAT CSA	138.91 (89.99)	205.84 (103.21)	0.012*
Staging IMAT CSA	8.97 (4.35)	12.56 (7.36)	0.013*
Staging total fat CSA	328.79	447.00	0.024*
Staging SMI	50.86 (10.63)	48.71 (9.00)	0.420
Staging SATI	60.39 (40.85)	77.20 (58.58)	0.173
Staging VATI	45.58 (29.61)	68.13 (33.23)	0.007*
Staging TATI	105.97 (65.78)	145.33 (66.81)	0.028*

Table 15 staging CT data for patients undergoing neoadjuvant chemotherapy by age

SMI measured in cm^2/m^2

CSA measured in cm^2

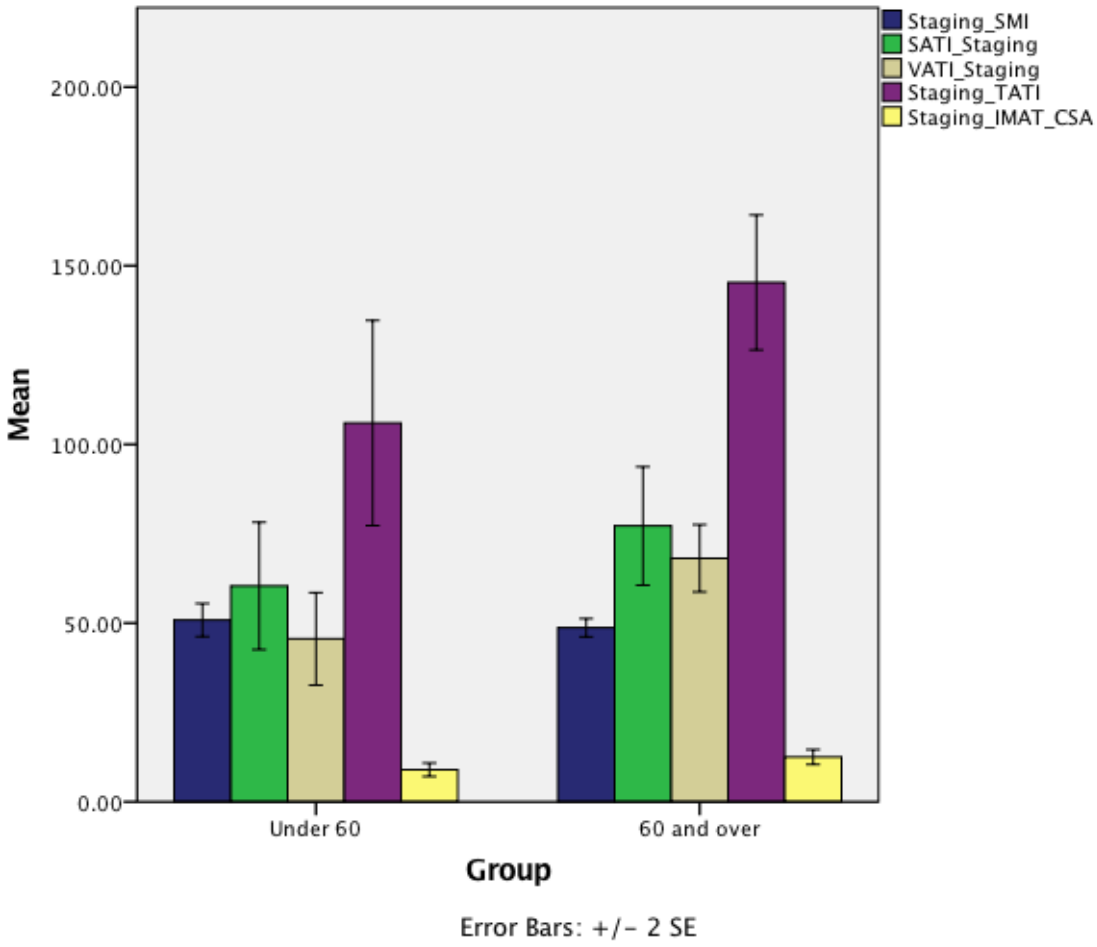


Figure 25 CT staging data by age in patients undergoing neoadjuvant chemotherapy

Older patients have lower SMI and higher adipose tissue indices

SMI measured in cm^2/m^2

	Sex		
	Male	**Female**	**P Value**
N	56	15	N/A
Weight	76.24 (24.67)	73.88 (24.95)	0.748
BMI	26.69 (5.26)	28.28 (6.78)	0.410
% weight loss at diagnosis	4.60 (5.57)	6.31 (7.05)	0.397
Staging muscle CSA	155.19 (29.08)	120.99 (21.75)	<0.01*
Staging SAT CSA	184.30 (97.32)	327.21 (233.67)	0.035*
Staging VAT CSA	201.15 (101.76)	129.63 (92.65)	0.016*
Staging IMAT CSA	11.77 (7.23)	10.49 (4.86)	0.426
Total fat CSA	397.22 (170.02)	467.33 (274.39)	0.359
Staging SMI	50.6 (9.8)	44.8 (6.9)	0.013*
Staging SATI	59.07 (31.05)	120.42 (87.22)	0.017*
Staging VATI	65.15 (32.85)	47.68 (34.01)	0.089
Staging TATI	124.07 (54.15)	169.60 (100.85)	0.111

Table 16 Staging CT data for patients undergoing neoadjuvant chemotherapy by sex

SMI measured in cm^2/m^2

CSA measured in cm^2

Eleven patients failed to complete all cycles and 73% did not receive any dietetic intervention or had no dietetic intervention noted. Mean CT interval was 88 days. Chemotherapy and pathology details are shown in table 17.

Patient characteristics	Number of patients
Chemotherapy regimens and tolerance:	
ECF – 4/3/2/1 cycles	0/2/0/0
ECX – 4/3/2/1 cycles	5/4/1/0
Cis5FU – 2/1 cycles	49/8
Carboplatin & Taxal	1
Unknown (chemotherapy undertaken in another region)	1
Dietetic intervention during NAC:	
None/not documented	52
Nutritional oral supplements	5
NG Feed	14
Stage:	
T4 N2/3	4
T4 N0/1	0
T3 N2/3	19
T3 N0/1	36
T2 N2/3	2
T2 N0/1	4
T1 N2/3	2
T1 N0/1	5

Table 17 Details of chemotherapy regimens, nutritional supplementation and final pathology

Both age groups had a net overall loss of muscle following NAC however the amount of muscle lost and therefore the changes in muscle CSA and SMI were not significantly different (-7.71 v -13.00 cm^2 and -2.67 v -4.56 cm^2/m^2, p=0.398 and 0.380). Older patients (those >60 years) lost significantly more adipose tissue than their younger counterparts with significant changes in VAT CSA (7.66 v -17.75 cm^2, p=0.014) and VATI (2.57 v -5.42 cm^2/m^2, p=0.026), SATI (-1.15 v -12.72 cm^2/m^2, p=0.035), total adipose CSA (5.95 v -45.46 cm^2, p=0.004) and TATI (5.87 v -47.83 cm^2/m^2, p=0.004) seen. An overall net gain in adipose tissue CSA was seen in those under 60 years (table 18, figures 26 and 27).

	Age (years)		
	<60	**60 and over**	**P Value**
Change in muscle CSA	-7.71 (21.07)	-13.00 (29.51)	0.398
Change in SAT CSA	-7.10 (26.10)	-31.78 (85.59)	0.069
Change in VAT CSA	7.66 (28.52)	-17.75 (55.62)	0.014*
Change in IMAT CSA	0.30 (3.79)	2.38 (10.43)	0.222
Total fat loss CSA	5.95 (45.26)	-45.46 (101.75)	0.004*
Change in SMI	-2.67 (6.89)	-4.56 (10.69)	0.380
Change in SATI	-1.15 (9.16)	-12.72 (35.19)	0.035*
Change in VATI	2.57 (9.42)	-5.42 (20.09)	0.026*
Change in TATI	5.87 (45.06))	-47.83 (105.56)	0.004*

Table 18 Change in tissue indices and area by age following neoadjuvant chemotherapy

SMI measured in cm^2/m^2
CSA measured in cm^2

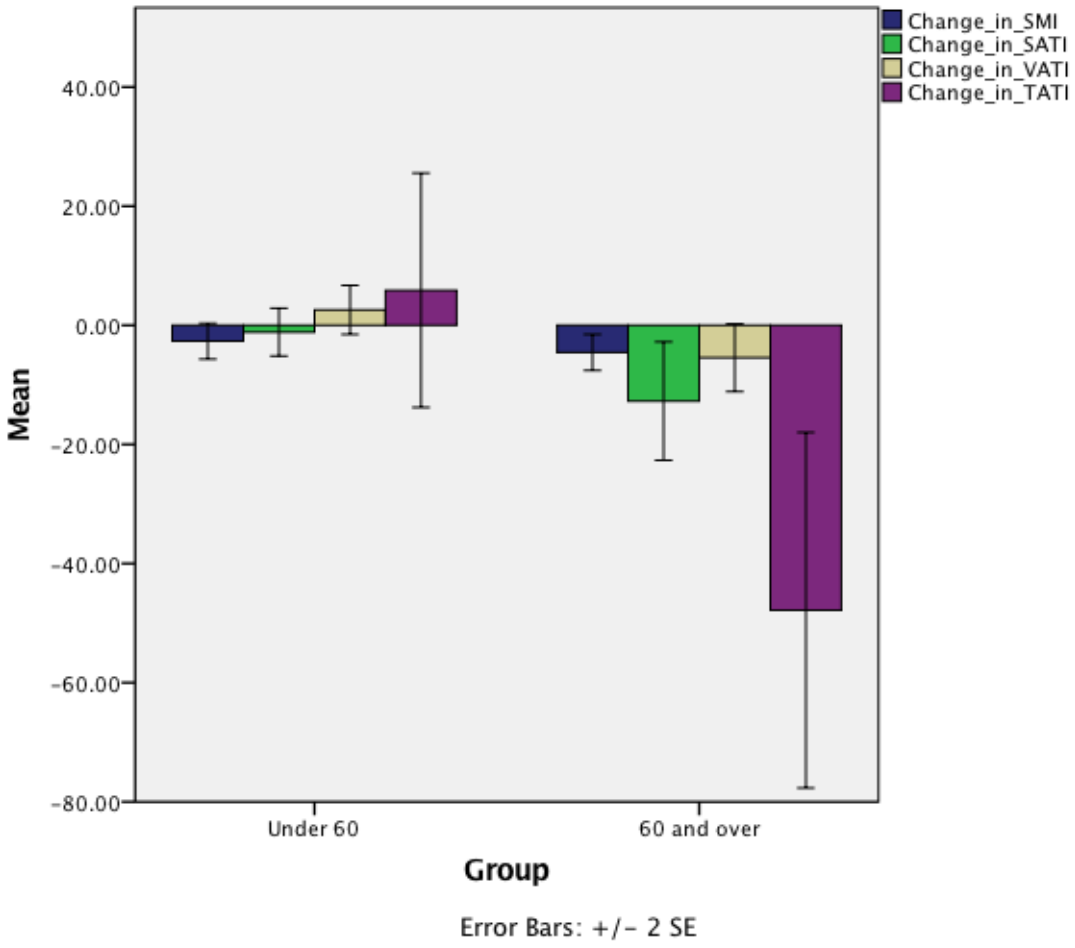


Figure 26 Change in tissue index following neoadjuvant chemotherapy by age

Both age groups lost muscle mass. Those under 60 gained adipose tissue whereas those over 60 had a net loss of all types of adipose tissue

SMI measured in cm^2/m^2

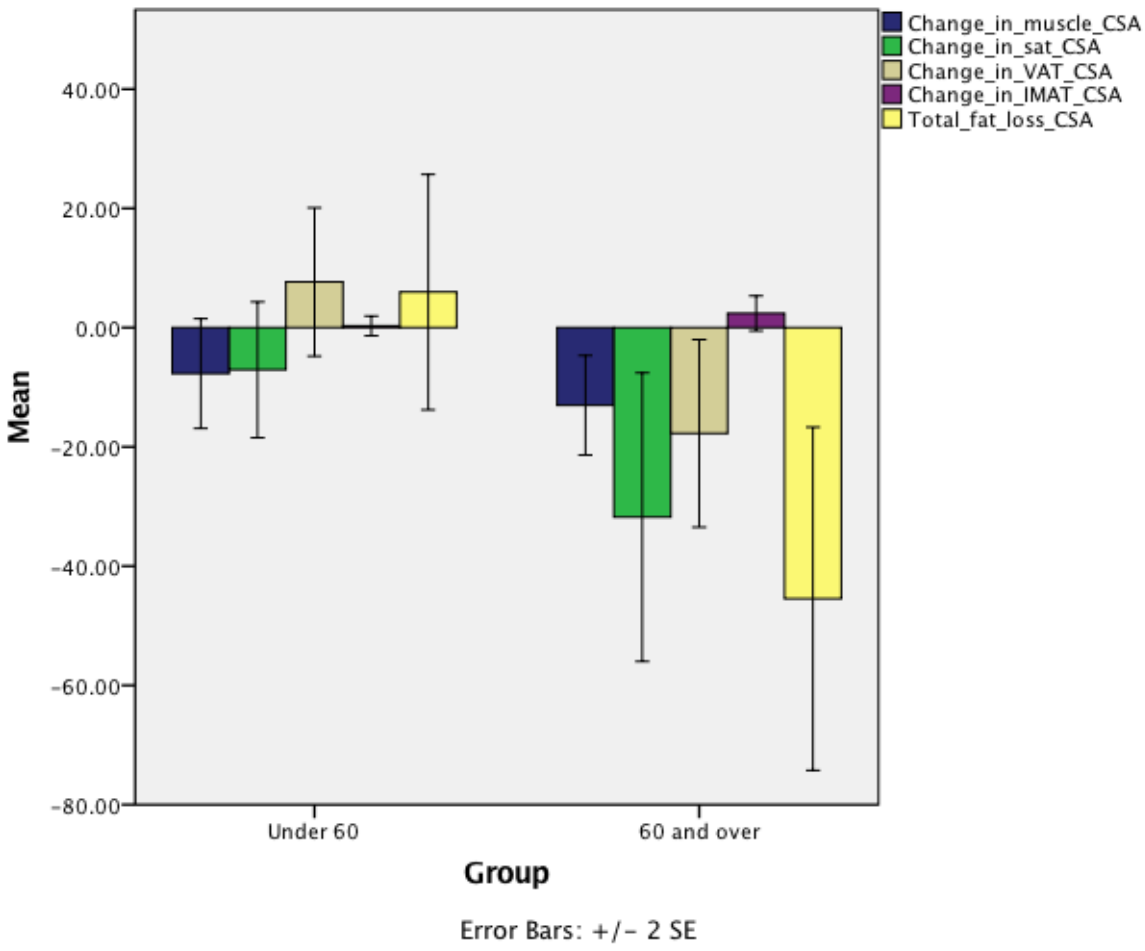


Figure 27 Change in tissue area following neoadjuvant chemotherapy by age

Both age groups lost muscle mass. Those under 60 gained adipose tissue whereas those over 60 had a net loss of all types of adipose tissue

CSA measured in cm^2

Following NAC both sexes lost muscle mass but this was not significantly different (-11.23 v – 12.24 cm^2 for muscle CSA and -3.86 v -4.56 cm^2/m^2 for SMI, p=0.875 and p=0.76 respectively). Females lost significantly more subcutaneous adipose tissue (SATI: -3.25 v -31.90 cm^2/m^2, p=0.05) and had a higher overall total fat loss (-11.38 v -100.71 cm^2 for total adipose CSA and -13.38 v -101.26 cm^2/m^2 for TATI, p=0.008 and p=0.006 respectively). This data is summarized in table 19 and figures 28 and 29.

	Sex		P Value
	Male (n=56)	**Female (n=15)**	
Change in SMI	-3.86 (10.32)	-4.56 (7.27)	0.76
Change in SATI	-3.25 (16.50)	-31.90 (53.24)	0.05*
Change in VATI	-1.14 (15.37)	-10.21 (24.71)	0.194
Change in TATI	-13.38 (83.39)	-101.26 (105.41)	0.008*
Change in IMAT CSA	2.08 (10.00)	0.55 (3.38)	0.339
Change in muscle CSA	-11.23 (29.12)	-12.24 (19.54)	0.875
Change in SAT CSA	-11.05 (47.84)	-74.59 (121.87)	0.066
Change in VAT CSA	-6 (45)	-27 (66)	0.263
Total fat loss	-11.38 (78.87)	-100.71 (103.81)	0.006*

Table 19 Change in body composition following neoadjuvant chemotherapy by sex

SMI measured in cm^2/m^2
CSA measured in cm^2

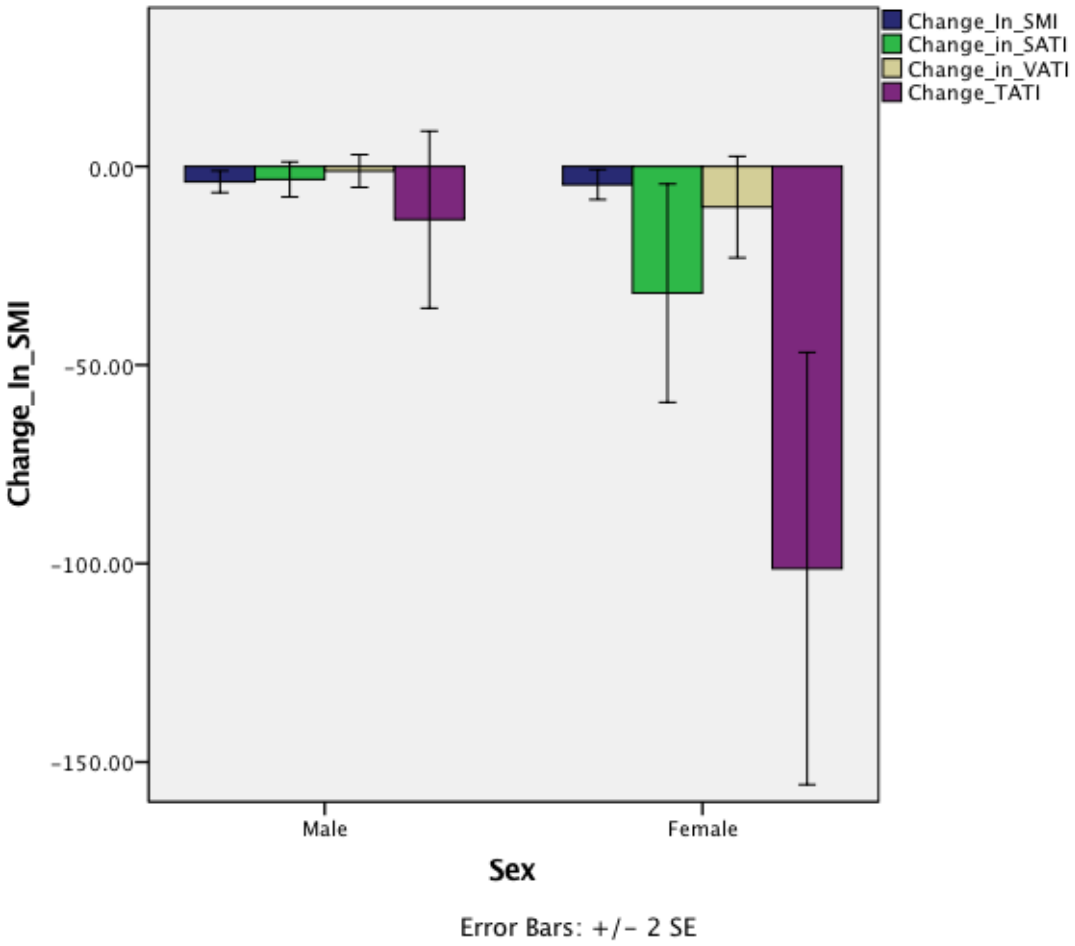


Figure 28 Change in tissue index following neoadjuvant chemotherapy by sex

Both sexes lost muscle mass. Females had greater losses of all types of adipose tissue

SMI measured in cm^2/m^2

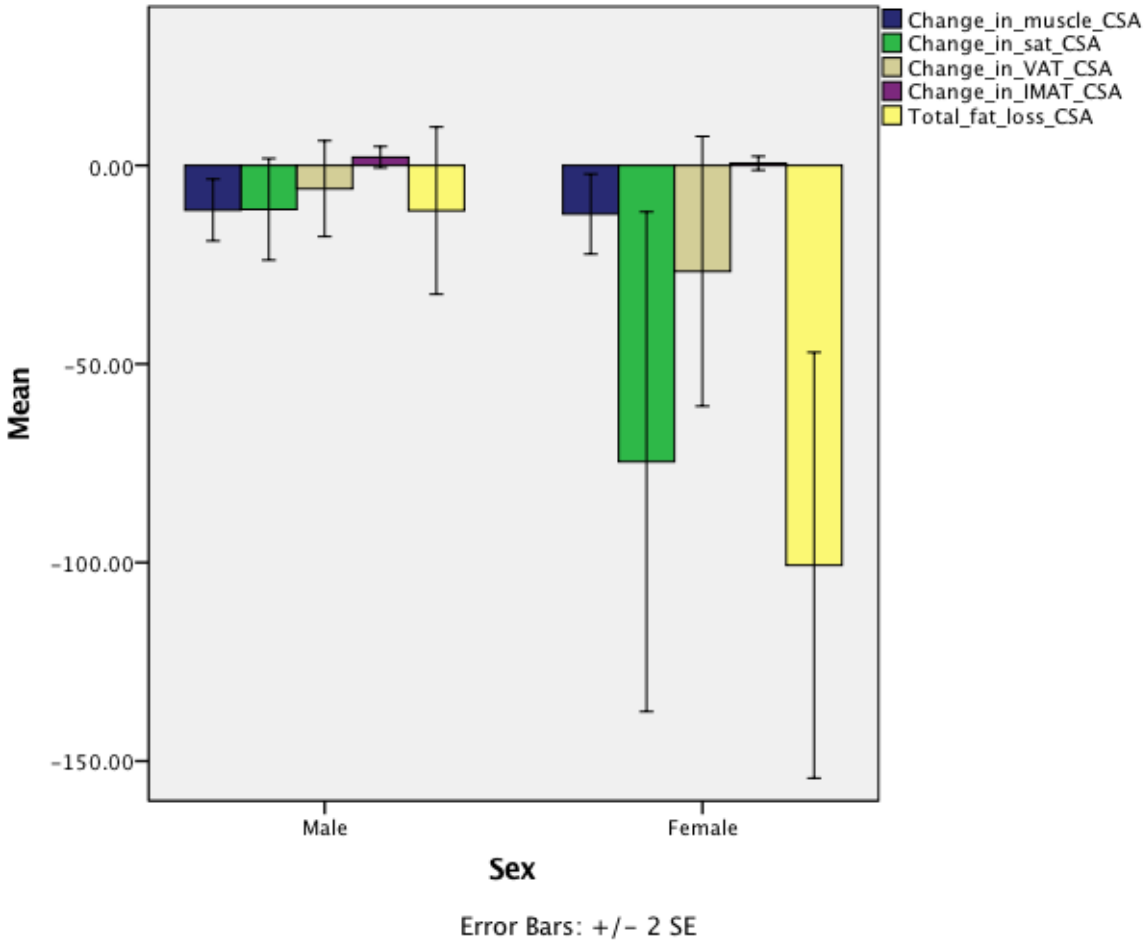
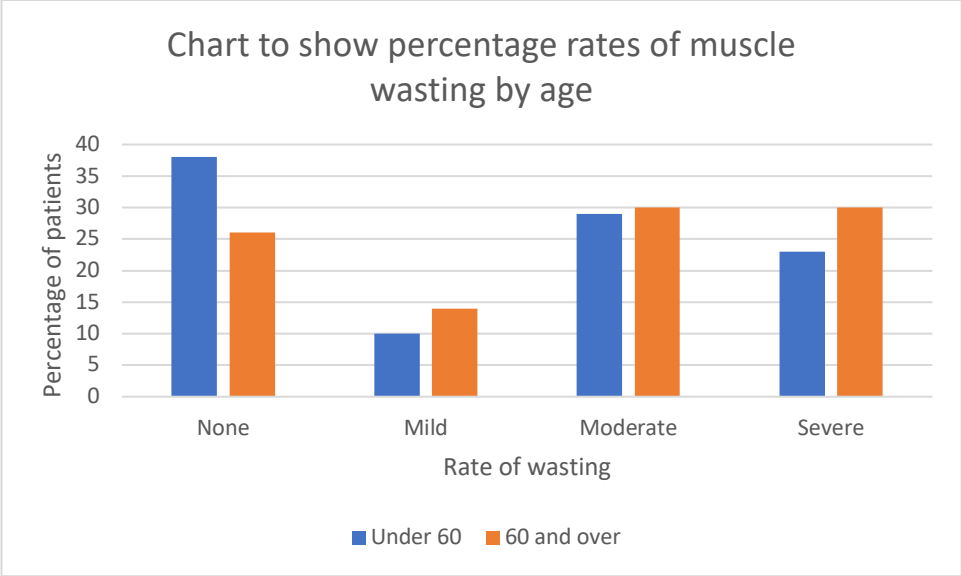


Figure 29 Change in tissue area following neoadjuvant chemotherapy by sex

Both sexes lost muscle mass. Females had greater losses of all types of adipose tissue

CSA measured in cm^2

Rates of muscle wasting did not significantly vary by age (p=0.122) whereas rates of adipose wasting did (p<0.001) as shown in figure 30.



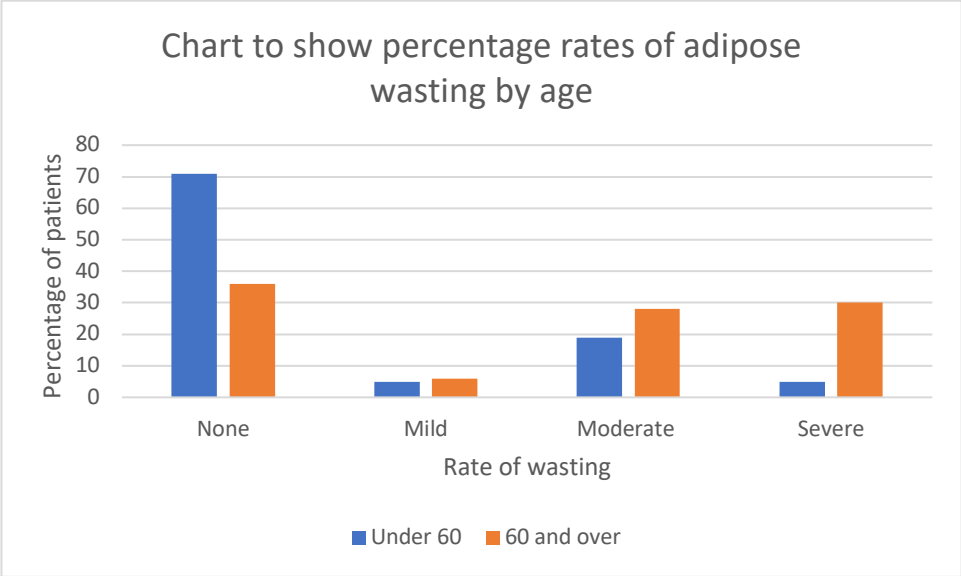


Figure 30 percentage rates of tissue wasting by age

Older patients demonstrate more severe rates of both muscle and adipose wasting

Those patients who had a more severe rate of muscle wasting also demonstrated a greater loss of total body fat [-86.88 cm^2/m^2 (123.17) in the severe wasting group, p=0.010]. Those patients with a severe rate of adipose wasting also demonstrated the largest change in SMI [-8.09 cm^2/m^2 (13.67) in the severe wasting group, p=0.026] as shown in figure 31.

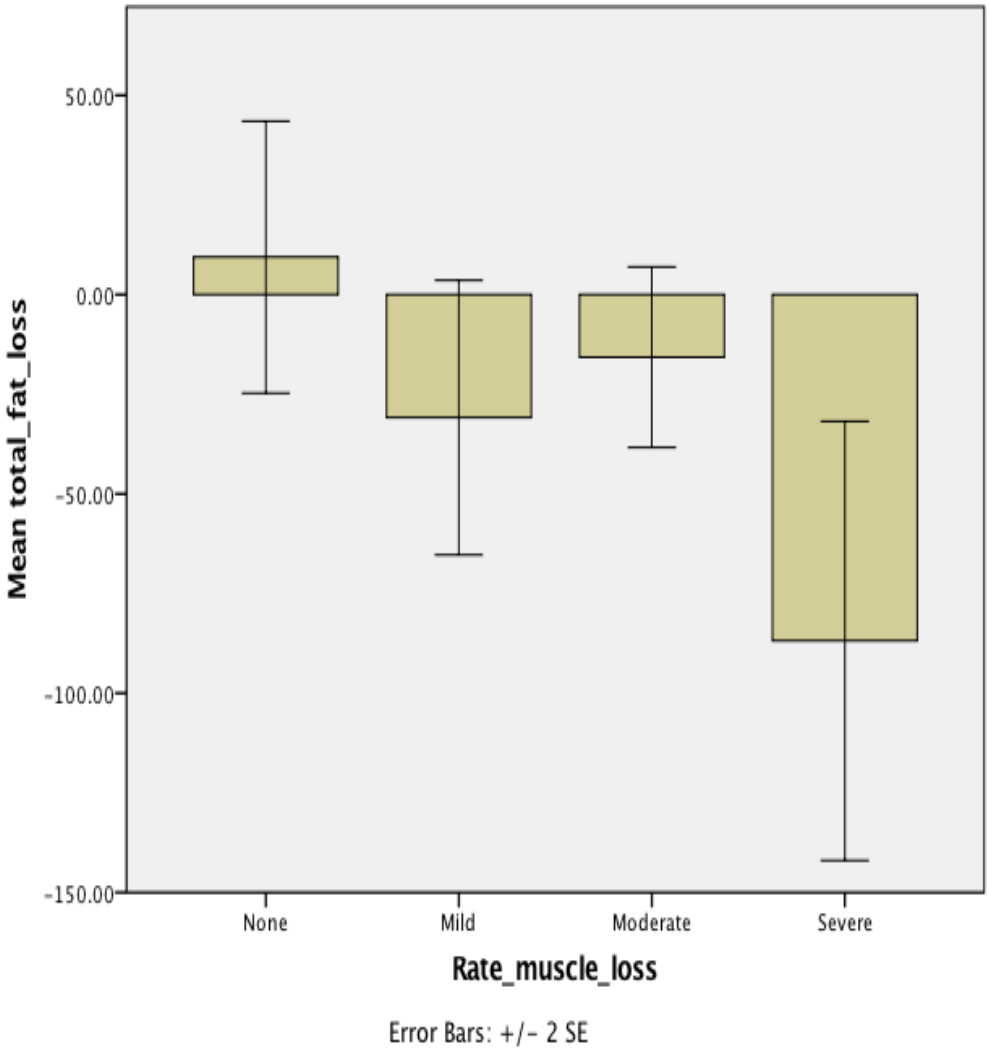


Figure 31 Association between adipose tissue and muscle loss

Those patients who had the most severe rate of muscle loss also had the largest loss of total adipose tissue. Those who did not complete chemotherapy had the largest loss of muscle mass (p=0.006) and gain in IMAT (p=0.001). Changes in fat mass between the two was not significantly different (figure 32).

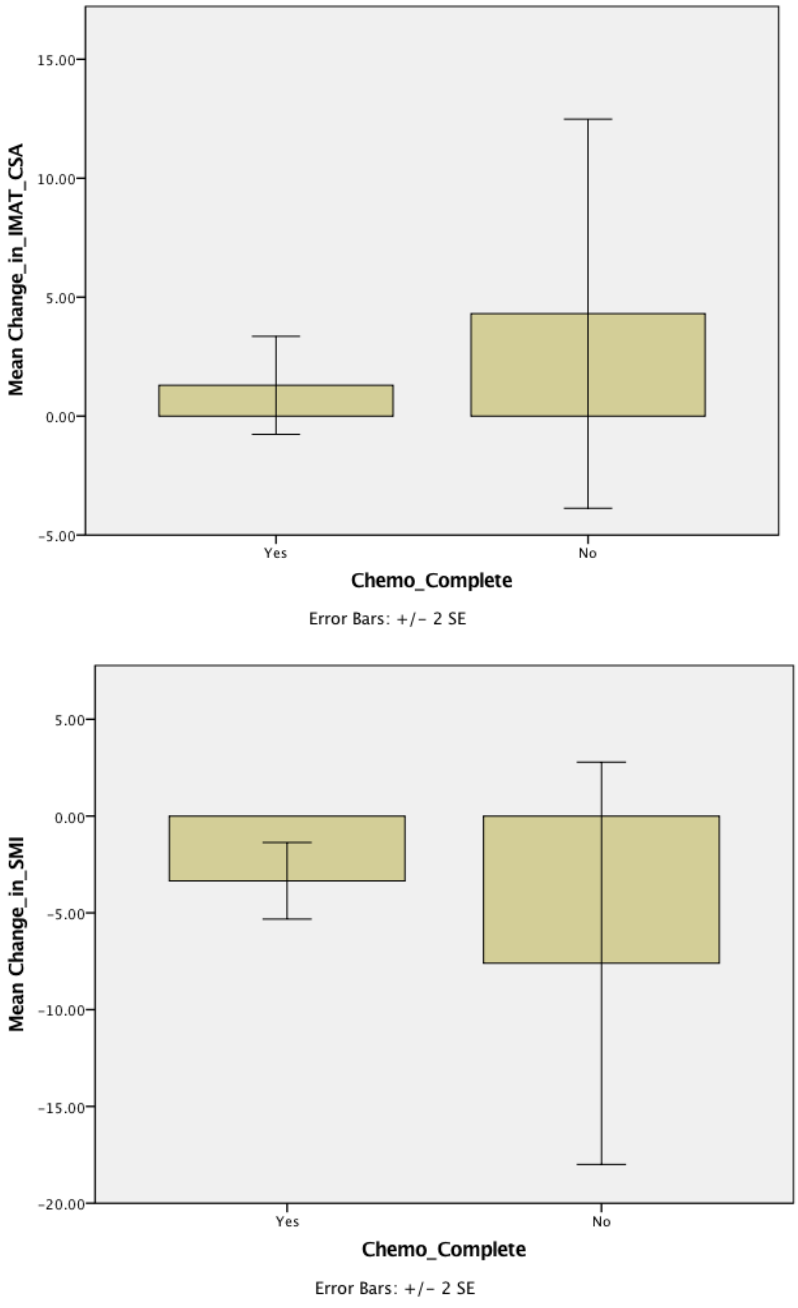


Figure 32 Changes in SMI and IMAT in those who did and did not complete chemotherapy
Those who did not complete chemotherapy had higher loss of SMI and gain of IMAT. SMI measured in cm^2/m^2 , IMAT CSA measured in cm^2

4.5 Discussion

In this retrospective cohort study the significance of differences in adipose and skeletal muscle mass by age group and sex were investigated in patients with potentially curable oesophageal cancer. Females had a lower baseline muscle mass but interestingly there were no differences in baseline muscle mass by age. Those who failed to complete chemotherapy showed the largest losses of muscle mass indicating they may be a particularly vulnerable patient group. Adipose depots differed in terms of baseline mass and predisposition to wasting. Older patients had a higher baseline adipose mass but also demonstrated a greater amount of adipose wasting following chemotherapy. Females also showed a greater loss of subcutaneous adipose tissue in particular compared to males. Interestingly all patients demonstrated loss of muscle mass but this did not appear to be age or gender specific. To our knowledge this is the first study to examine the association between age, sex, body composition and oesophageal cancer.

BMI which is frequently used as an easy method of body composition assessment does not differentiate between fat and fat-free mass. Despite wide variations between tissue areas and indices in this study, BMI was never shown to be different between age groups or sex. Any changes in body composition are therefore unlikely to be detected by clinicians and an understanding of the type of tissue being lost is required to associate accumulation or loss of specific tissues with health outcomes. Visceral and

subcutaneous adipose tissue distribution was found to differ between age groups and males and females. Patients over the age of 60 had greater loss of visceral, subcutaneous and total adipose tissue compared with those under 60 who gained adipose mass following chemotherapy. Females also demonstrated a greater loss of subcutaneous and total adipose tissue. Visceral and subcutaneous depots differ in their metabolic activity with visceral adipose being shown to produce more inflammatory cytokines and adipokines therefore contributing to the inflammatory state found in cachexia (291). Subcutaneous adipose is a large producer of leptin which may confer benefits associated with insulin sensitivity, glucose and lipid metabolism (292). The impact of these differences in patients with cancer remain relatively unexplored but they highlight the need to evaluate cancer mortality not just on the basis of muscle wasting or total adiposity.

Animal models have shown that fat loss occurs prior to muscle loss. Mice who were injected with lung carcinoma or melanoma cells showed fat loss occurred prior to muscle loss at early stages of tumour growth and that the rate of wasting was greater than that of muscle (159). Patient based studies have shown losses of subcutaneous and visceral fat following chemotherapy in colorectal and lung cancer and a further study in pancreatic cancer also indicated that the severity of wasting rather than the amount of visceral adipose tissue lost was a better indicator of survival (293). These patients also lost subcutaneous tissue at a similar rate but this was not related to outcome.

The rate of adipose tissue change was also much greater than that of muscle. The only other study undertaken in oesophagogastric patients demonstrated that patients with locally advanced cancers lost on average 6% fat mass following NAC (193). Little is known in general about the effects of chemo or radiotherapy which may induce alterations in body composition. Potential differences in fat loss however, have not been consistently demonstrated due to variations in the type of scans used in various studies (e.g. CT/DEXA) as well as differences in tumour type and stage (resectable or palliative). Studies also differ in the way they report fat mass – either by CSA or indexed for height. This study used both to allow for maximal comparison with others. Regression equations have also not yet been developed for adipose tissue in cancer however visceral adipose CSA at L3 has been shown to correlate with total body visceral adipose tissue volume in healthy controls (294).

This was an observational study of changes in body composition and as such was not powered to detect differences in clinical outcomes. Patients in this study were chosen as they had been previously recruited to cachexia studies and had undergone muscle biopsy. They were therefore felt to be representative of the target population thus eliminating the risk of selection bias. There were large differences in numbers between age groups but this represents “real life” clinical practice. The risk of being diagnosed with oesophageal cancer is less in those under 50 and those over 80 are less likely to be considered suitable for radical treatment. Larger studies are required to

identify the effects of changes in body composition following chemotherapy on surgical outcomes. It is also important to note that the association between changes in tissue mass and chemotherapy does not necessarily imply causality.

4.6 Conclusion

Alterations in fat mass between age groups and sexes appear to be significantly different. Little is known about the prognostic significance of these depots in large populations and it therefore requires investigation throughout cancer disease progression. Due to the role of adipose tissue in controlling aspects of metabolism further identification of mediators and mechanisms of fat loss in cancer would be helpful to identify those who would benefit from early therapeutic intervention.

MECHANISMS OF CANCER CACHEXIA

Chapters 5,6 and 7

CHAPTER 5

NEUROMUSCULAR JUNCTIONS ARE STABLE IN CANCER CACHEXIA

5.1 Chapter overview

The forth chapter has highlighted the importance of muscle wasting by showing that all patients with cachexia regardless of age or sex demonstrate loss of muscle mass. This fifth chapter moves on to investigate a possible mechanism of muscle loss involving the neuromuscular junction (NMJ). Despite sharing pathophysiological features with a number of other neuromuscular wasting conditions, the mechanisms underlying cachexia remain poorly understood. Studies using animal models have suggested that pathological targeting of the NMJ may play a key role in the pathogenesis of cachexia, but this has yet to be investigated in human patients.

5.1.1 Objectives

To determine if the NMJ plays a role in muscle wasting in cancer.

5.1.2 Methods

High-resolution morphological analyses were undertaken on NMJs in samples of rectus abdominis muscle obtained from patients undergoing surgery for gastrointestinal cancer and compared with control cases (N=30 individuals; n=1165 NMJs). Cancer patients included those with both cachexia (according to the consensus definition) and weight stable disease.

5.1.3 Results

Despite low SMI and a significant degree of muscle fibre atrophy noted in patients with cachexia, compared to weight stable cancer and control patients, NMJ morphology was fully conserved, with no significant differences observed in any of the major pre- and post-synaptic variables measured (including axon diameter, nerve terminal area and complexity, motor endplate area and fragmentation, and percentage overlap between pre- and post-synaptic structures).

5.1.4 Conclusion

NMJs remain structurally intact during cancer and cachexia, suggesting that denervation of skeletal muscle is not a major driver of disease pathogenesis. The absence of NMJ pathology in human cancer is in contrast to observations noted in animal models, and supports the hypothesis that intrinsic changes within skeletal muscle, independent of any changes in motor neurons, represent the primary locus of pathology in cachexia.

5.2 Introduction

At present, the consensus definition of cancer cachexia confirms loss of skeletal muscle as a key feature of the condition (28). Cancer cachexia is largely mediated by pro-inflammatory cytokines and tumour associated mediators, which results in the activation of catabolic pathways in skeletal muscle (295). In this regard, cancer cachexia shares many of the muscle-specific and systemic inflammatory pathways common to the muscular dystrophies (49,296,297).

Although the majority of cachexia research to date has focused on muscle abnormalities as the major locus of pathophysiology, many lines of evidence have implicate NMJs a critical and early mediator of neuromuscular dysfunction and breakdown, as denervation represents a common, key hallmark of several, related muscle wasting conditions and neuromuscular diseases (298–301). For example, NMJ pathology is considered to represent an early and key driver of neuromuscular defects in age-related sarcopenia (302–304), driven at least in part by an age related loss of lower motor neurons and motor axons (305). As cachexia and sarcopenia share similar molecular mechanisms (306) and as cachexia is considered to be a multifactorial syndrome that includes components of both age-related sarcopenia and bed rest/reduced physical activity (289), the NMJ has similarly been implicated in the pathogenesis of cachexia. Moreover, the identification of displaced mononuclei in the muscle of cachectic patients and C26 tumour-bearing mice

has previously been used to suggest the presence of denervation (50). Furthermore, a member of the UPP pathway (MuRF1) that is pivotal to muscle wasting in tumour-bearing mice (and other murine models of muscle wasting) has been shown to be critical to the maintenance of NMJs (307). Additionally, recent mouse studies of mTOR signalling, a key regulator of protein synthesis that is suppressed by inflammatory mediators in cancer cachexia (308,309) have shown that muscle specific-deletion of mTOR or Raptor results in muscle fibrillation or NMJ fragmentation.

The role of the muscle-nervous system interface in human cachexia is therefore of particular importance to both basic and clinical scientists, and relevant to both our understanding of disease pathophysiology and the development of effective treatments. Previous studies in patients with heart failure and cardiac cachexia have shown good correlation between body composition and levels of C-terminal fragment of agrin, a proteoglycan important for forming NMJs (310). Similarly, cancer cachexia has been the subject of co-culture/informatics projects (311) and ongoing patient studies (see ClinicalTrials.gov identifier NCT03477721 “NUMANCAN study). Nevertheless, experimental evidence supporting a direct role for NMJs in human cachexia is highly reliant on studies of animal models. However, recent data have revealed striking and unexpected differences between the cellular and molecular anatomy of NMJs in humans compared to other model

organisms (e.g. mice (46)), suggesting that findings from animal models may not be directly applicable to human patients.

The present study builds upon my collaborators recent work establishing a robust protocol to facilitate sampling and high-resolution, quantitative morphological analyses of NMJs from human patients undergoing surgery. These protocols have been adapted to facilitate a comprehensive analysis of the NMJ in cancer cachexia patients. Findings reveal that, in stark contrast to predictions from animal models, NMJs remain structurally intact and conserved during cancer and cachexia, suggesting that denervation of skeletal muscle is not a major driver of disease pathogenesis.

5.3 Methods

5.3.1 Patient recruitment

Patients with a confirmed diagnosis of gastrointestinal cancer (oesophageal, gastric or colonic) suitable for surgical resection with curative intent were recruited from the regional multi-disciplinary team meeting (n = 20; Table 20) and comprised patients with both weight stable disease (n = 10) and cancer cachexia (n = 10) based on consensus definition. Suitable age-matched control patients undergoing a range of elective abdominal procedures (e.g. repair of aortic aneurysm or donor nephrectomy) were also approached prior to surgery (n = 10; Table 20). Inclusion criteria included age over 18 and the

ability to give consent. Exclusion criteria for the control cohort included previous history of malignancy.

5.3.2 Body composition analysis

SMI was calculated from routine staging CT performed prior to surgical intervention as previously described. Those patients who underwent NAC (n=5 in the cachectic group and n=3 in the weight stable group) had their post chemotherapy re-staging scan analysed. SMI cut-offs for low SMI were obtained from previously published reference data. Cachexia was defined according to standard consensus (>2% weight loss and low muscularity;) as this was the only definition, which has been shown to be associated with histological muscle wasting in previous studies (312).

5.3.3 Tissue sampling

Tissue sampling was performed under general anaesthesia at the start of the surgical procedure. All samples were obtained approximately 4 to 6 weeks following the cessation of chemotherapy. Biopsies of rectus abdominis (RA) muscle were obtained following initial opening of the abdomen. Rectus abdominis has an 'in-series' architecture, with motor endplate bands located throughout its length (figures 33 and 34) (313). To ensure successful sampling, a longitudinal strip of muscle lying between two contiguous tendinous intersections was obtained using sharp dissection (approx. 0.5 x 0.5 x 2.0 cm).

Samples were immediately fixed in 4% paraformaldehyde (PFA) for approximately 2 hours, followed by washing and storage in 1xPBS.

(226)

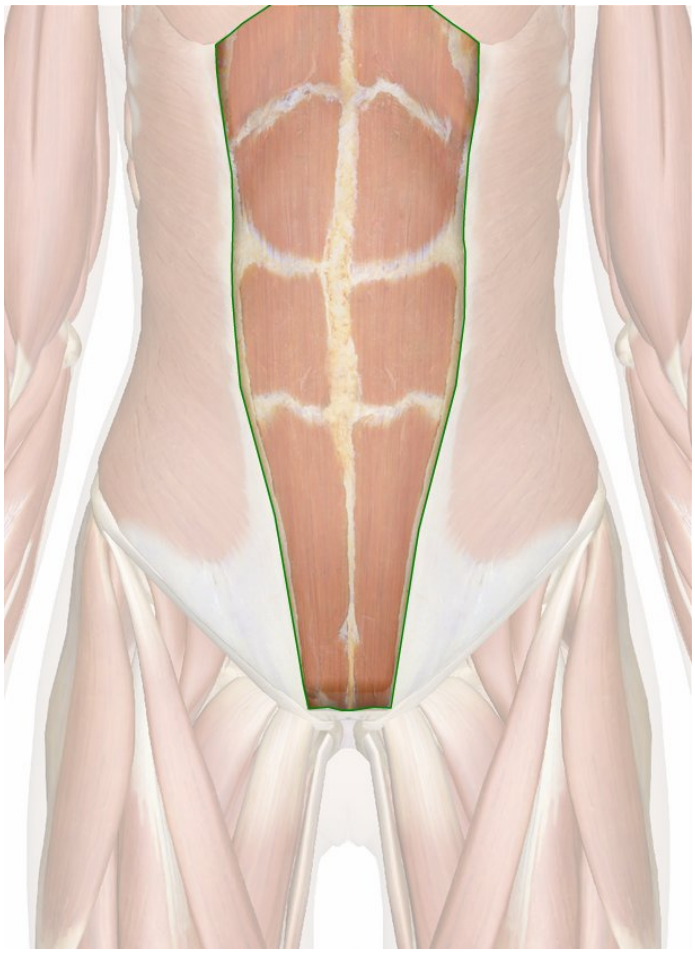


Figure 33 Anatomical distribution of rectus abdominus muscles

Rectus abdominus muscle area highlighted

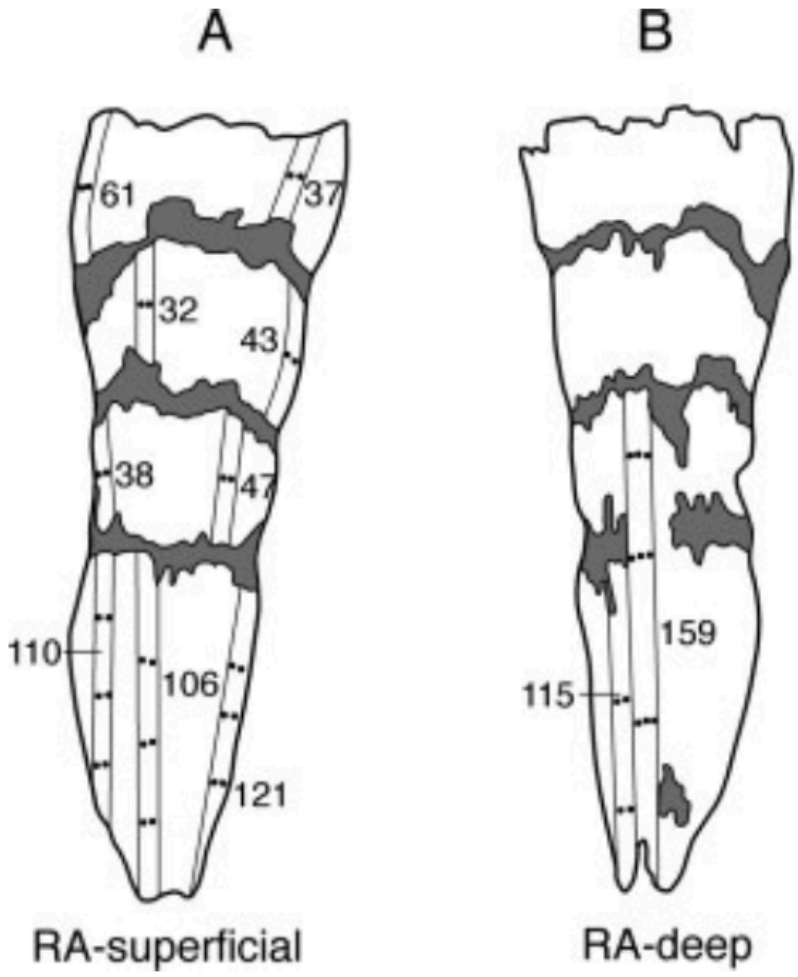


Figure 34 Differences between superficial and deep rectus

Tendinous intersections subdivide each rectus muscle into a series of smaller false muscles

5.3.4 Tissue processing and NMJ immunohistochemistry

Teased muscle fibre preparations were immunohistochemically labelled for NMJ analysis. In order to visualise key pre-synaptic proteins (the synaptic vesicle protein SV2 and the neurofilament protein 2H3), with post-synaptic acetylcholine receptors (AChRs) labelled using rhodamine-conjugated alpha-bungarotoxin. Optimal labelling was achieved with incubation in primary antibodies for 3 nights and secondary antibodies for 1 night. Samples were whole-mounted in Mowiol and stored at -20^{o}C prior to imaging.

5.3.5 Antibodies

Primary antibodies: mouse monoclonal SV2 and 2H3 (1:50; Developmental Studies Hybridoma Bank, University of Iowa). Secondary antibodies: AlexaFluor-488-conjugated donkey anti-mouse IgG antibody (1:200; A21202, Life Technologies). Rhodamine-conjugated alpha-bungarotoxin (2 ug/mL; BTIU00012, VWR International).

5.3.6 Confocal imaging and NMJ-morph analysis

Confocal images of individual en face NMJs and their pre-terminal axons were acquired on a Zeiss LSM 710 or Nikon A1R confocal laser-scanning microscope, using standardised imaging approaches (46,314). In addition, individual muscle fibre diameter measurements were obtained on an Olympus IX71 microscope and Hamamatsu C4742-95 camera with Openlab Improvision software. All images were then analysed according to a

standardised workflow ('NMJ-morph'), as previously described. NMJ-morph data output generates 21 separate morphological descriptors for each NMJ (see table 21), including pre- and post-synaptic variables (e.g. nerve terminal area, motor endplate area, etc.), derived variables (e.g. nerve terminal complexity, motor endplate fragmentation, etc.) and related nerve and muscle measurements (including motor axon diameter and muscle fibre diameter). As per NMJ-morph guidelines, a total of 40 NMJs were analysed for each individual patient/muscle sample (n = 18), where possible, giving a complete dataset of 697 NMJs. Parameters assessed by NMJ morph are summarised in figure 35. Overall methods are summarised in figure 36.

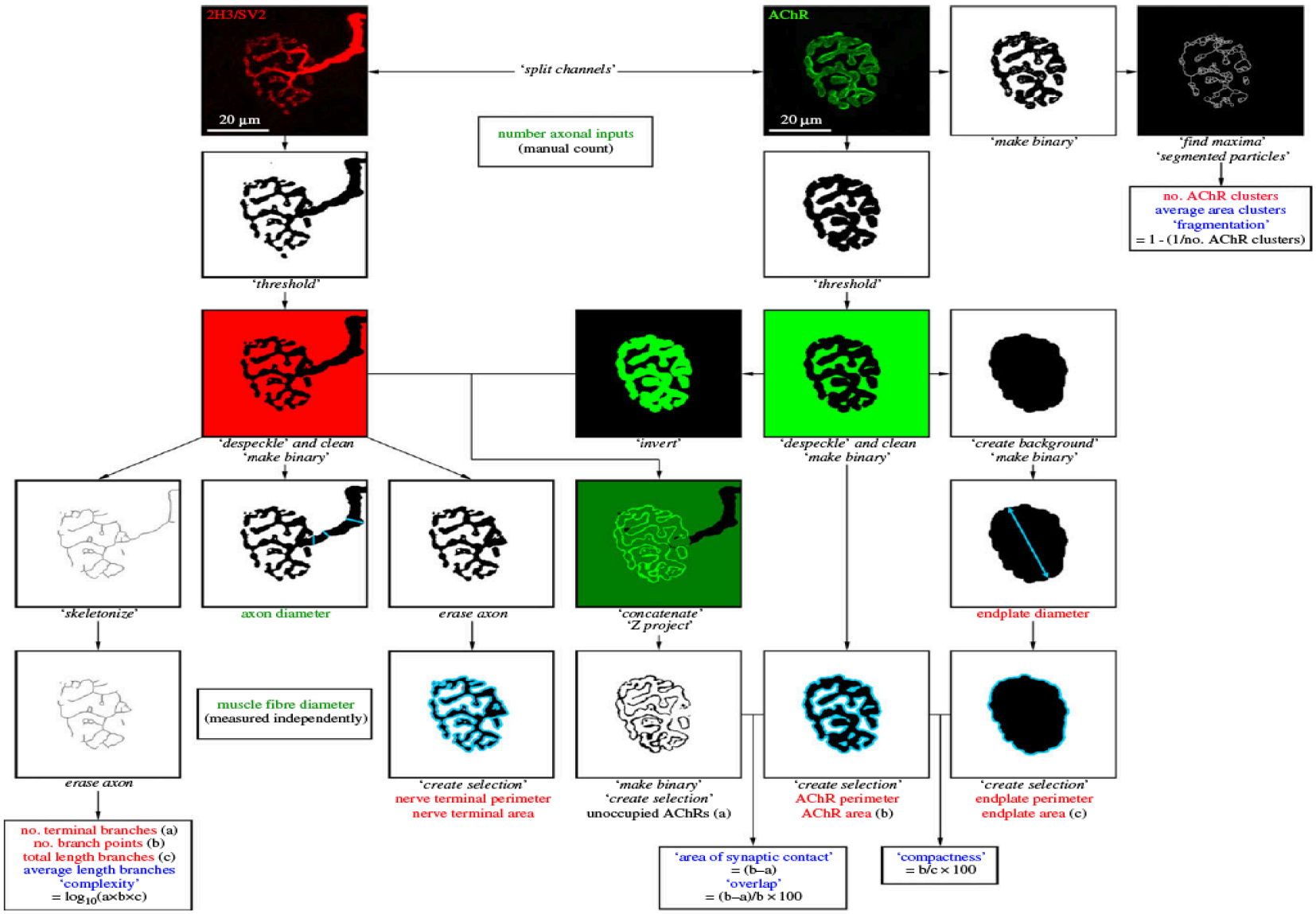


Figure 35 Parameters assessed by NMJ Morph. Stepwise process of analysis shown followed by recorded end outputs

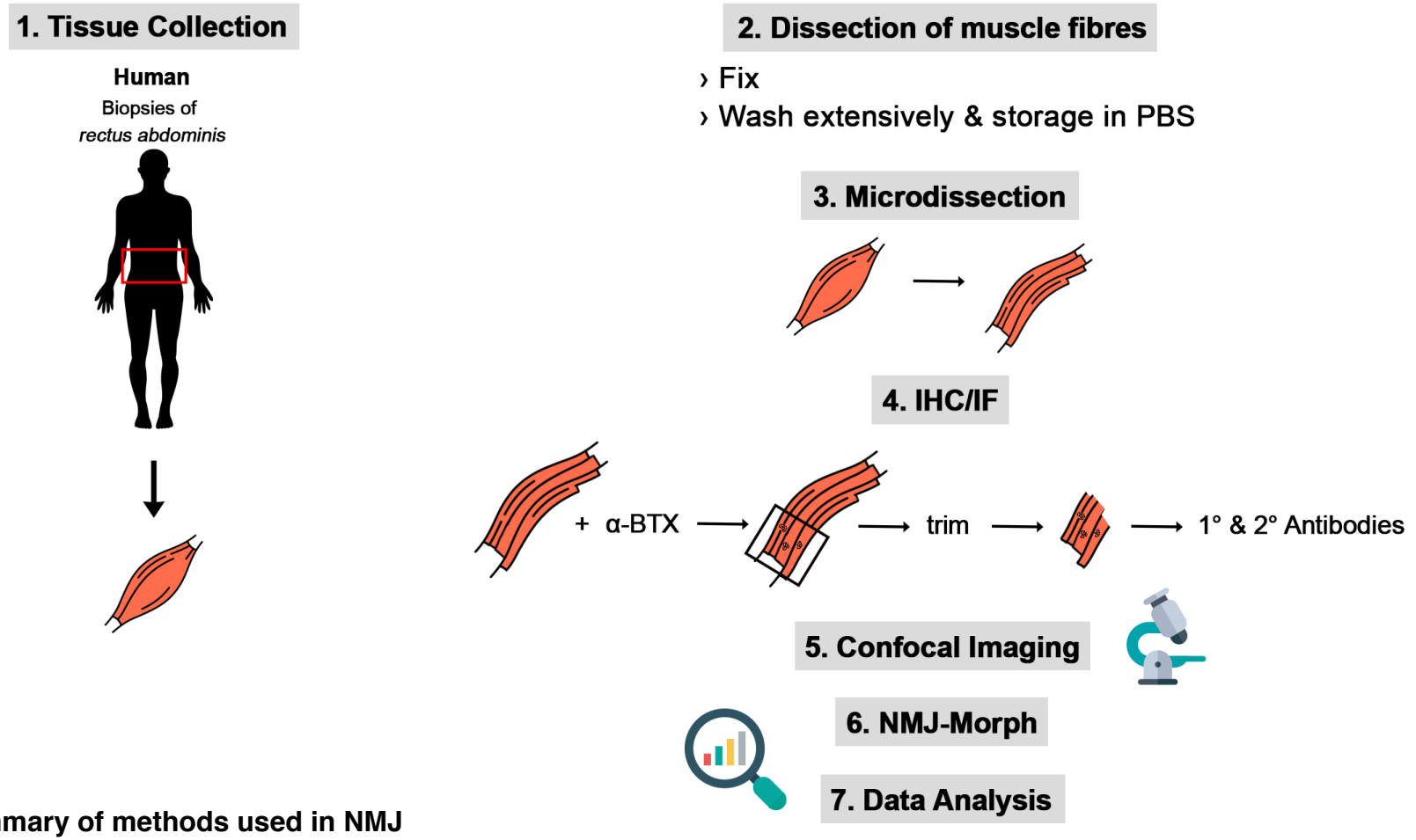


Figure 36 Summary of methods used in NMJ analysis Tissue is sampled and fixed. It is then microdissected and IHC undertaken. NMJs are then imaged and analysed by NMJ morph

IHC/IF were performed in collaboration with the University of Edinburgh Anatomy Department. Confocal imaging and NMJ-morph was performed by Ines Boehm, University of Edinburgh.

5.3.7 Statistical analysis

Statistical analyses were performed using GraphPad Prism 6 Software. Results are expressed as mean (± standard error of mean [SEM]). Group comparison of normally distributed data (based on D'Agostino-Pearson test, $P > 0.05$) was performed by one-way analysis of variance (ANOVA) and Tukey's post hoc test; non-parametric data was analysed by Kruskal-Wallis test and Dunn's post hoc test.

5.4 Results

To investigate the role of the NMJ in cancer cachexia, a comprehensive morphometric analysis of the NMJ in samples of rectus abdominis muscle obtained from patients undergoing surgery for gastrointestinal cancer was performed. This case series included 10 patients with cancer cachexia, 10 weight stable cancer patients and 10 controls. Patient demographic data is shown in Table 20. Mean ages were 64 years in the control group, 66 years in the weight stable group and 70 years in the cachexia group. Cachectic patients demonstrated significantly lower SMI by CT criteria compared with weight stable patients (Table 20). Cachectic patients with low SMI also demonstrated a trend towards lower subcutaneous adiposity and higher visceral adiposity compared with weight stable cancer patients. These two body composition phenomena are associated with worsened outcomes in cancer patients (234,315) further confirming the cachectic patients as a high-risk group. Two

patients in the weight stable cancer group exhibited a small degree of weight loss, but were not cachectic by the consensus definition (27,28).

Patient Group (n = 10)	Ctrl	Weight Stable	Cachexia	F (DFn, DFd) or Kruskal Wallis statistic	P	Mean Difference Ctrl vs Weight Stable	Mean Difference Ctrl vs Cachexia	Mean Difference Weight Stable vs Cachexia
Male:Female	9:1	7:3	8:2	/	/	/	/	/
Age (years)	64 ± 2.89 (min 49, max 81)	66 ± 3.89 (min 40, max 83)	68 ± 3.00 (min 49, max 78)	F (2, 27) = 0.3887	0.682	-1.90	-4.10	-2.20
BMI	28.68 ± 1.32 (min 20.0, max 36.2)	27.00 ± 1.78 (min 22.4, max 41.1)	25.69 ± 1.13 (min 20.4, max 31.8)	F (2, 27) = 1.096	0.349	1.69	3.00	1.31
% weight loss	0 % ± 0 % (min 0 %, max 0 %)	0.89 % ± 0.5 % (min 0 %, max 4.38 %)	7.99 % ± 1.65 % (min 2.70 %, max 19.1 %)	22.71	< 0.0001 ****	-3.400 %	-16.10 % ****	-12.70 % **
SMI	52.0 ± 2.08 (min 38.5, max 59.6)	56.9 ± 4.0 (min 43.7, max 78.5)	40.3 ± 1.75 (min 30.6, max 52.3)	12.98	0.0015 **	-2.100	11.10 *	13.20 **
VATI	64.4 ± 9.85 (min 15.8, max 111.6)	53.0 ± 16.6 (min 6.73, max 188.4)	73.7 ± 11.5 (min 33.5, max 134.0)	F (2, 27) = 0.644	0.5329	11.39	-9.342	-20.73
SATI	67.3 ± 14.0 (min 2.58, max 153.2)	60.3 ± 11.3 (min 18.32, max 132.3)	55.7 ± 4.53 (min 39.1, max 82.1)	0.281	0.8688	1.900	1.700	-0.2000
Cancer type: **Oesophageal ACC** **Oesophageal SCC** **Oesophageal undifferentiated** **Gastric ACC** **Colonic ACC**	/	4 1 0 4 1	7 1 1 1 0	/	/	/	/	/
ECOG	/	0 (min 0, max 1)	0 (min 0, max 1)	/	/	/	/	/

Table 20 NMJ Patient demographic data Data are mean ± SEM

To confirm/validate the patient groupings based on the clinical and radiological guidelines (% weight loss and SMI, respectively) muscle fibre diameters in rectus abdominis from the three groups were assessed. Mean muscle fibre diameter was found to be significantly reduced (by almost 20%) in the cachectic patients compared with both controls and those with weight stable disease ($p < 0.0001$; Figure 37). These observations are in keeping with previously published data showing marked muscle fibre atrophy in cachexia (312,316) and support the patient group allocations based on consensus definition.

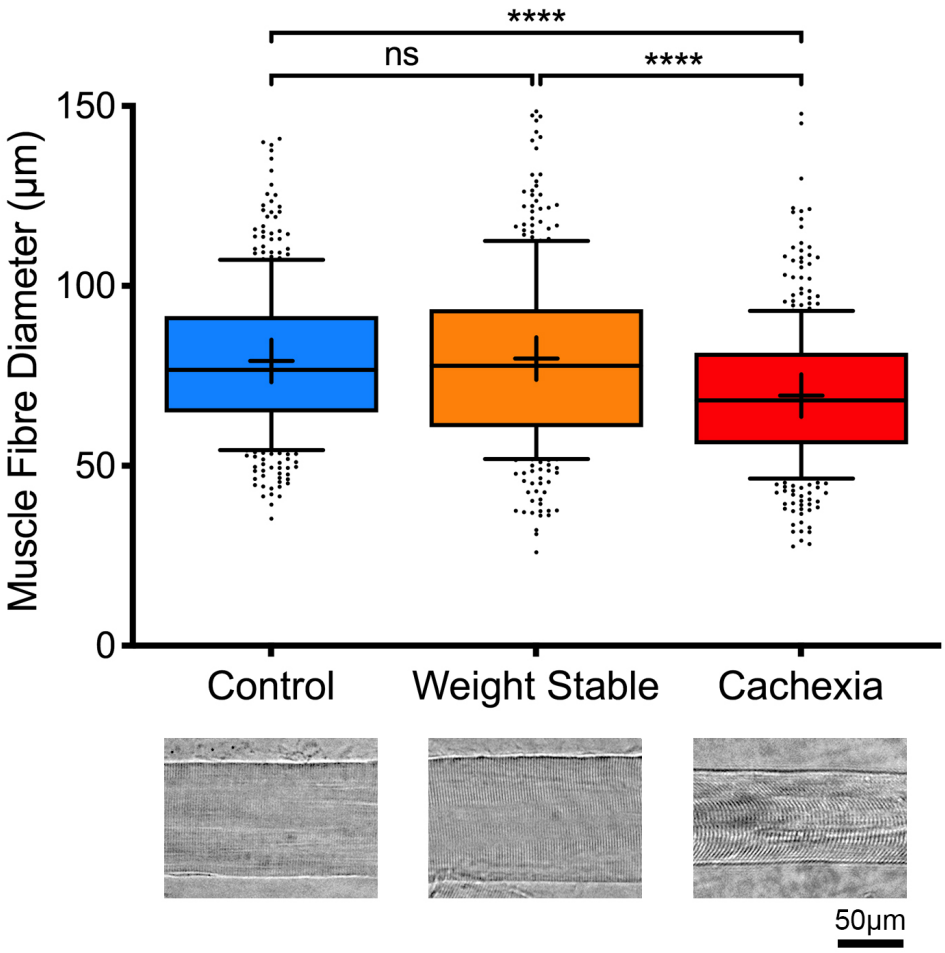


Figure 37 Atrophy of skeletal muscle fibres in cancer cachexia

Upper panel shows a box and whisker plot of muscle fibre diameters (MFD) in control (n = 10 patients), weight stable (n =10) and cachectic (n = 10) patients. Bottom panels are representative micrographs of single, teased muscle fibres from control (left), weight stable (middle) and cachectic (right) patients. Scale bar = 50µm. Cachectic patients had significantly reduced muscle fibre diameters compared with weight stable and control cases. Each group is the pooled data from ~40 measurements of muscle fibre diameter in each of 10 patients (control n = 388, weight stable n = 362, cachexia n = 400 muscle fibres in total). The box contains the mean (+) and median (line) muscle fibre diameters for the group and encloses the central 90% of the data; the whiskers represent the SEM; outlying data points are shown beyond the whiskers. One-way ANOVA paired with a Tukey's post-hoc test (****$P < 0.0001$).

Muscle fibre measurements were performed by Dr Ross Jones, University of Edinburgh Anatomy Department.

Given that muscle fibre pathology/atrophy would be predicted to result from and/or lead to NMJ instability based on findings from animal studies, an initial qualitative assessment of NMJs across the three human patient groups was performed. Despite the presence of muscle fibre atrophy in the cachectic patients, NMJ morphology was indistinguishable from that observed in weight stable and control patients (Figure 38). NMJs of all three cohorts were noted to display the typical 'nummular' morphology characteristic of human NMJs, and despite the predicted heterogeneity in form across the complete pool of NMJs (Figure 39), there was no evidence of gross pathological changes or denervation of skeletal muscle fibres.

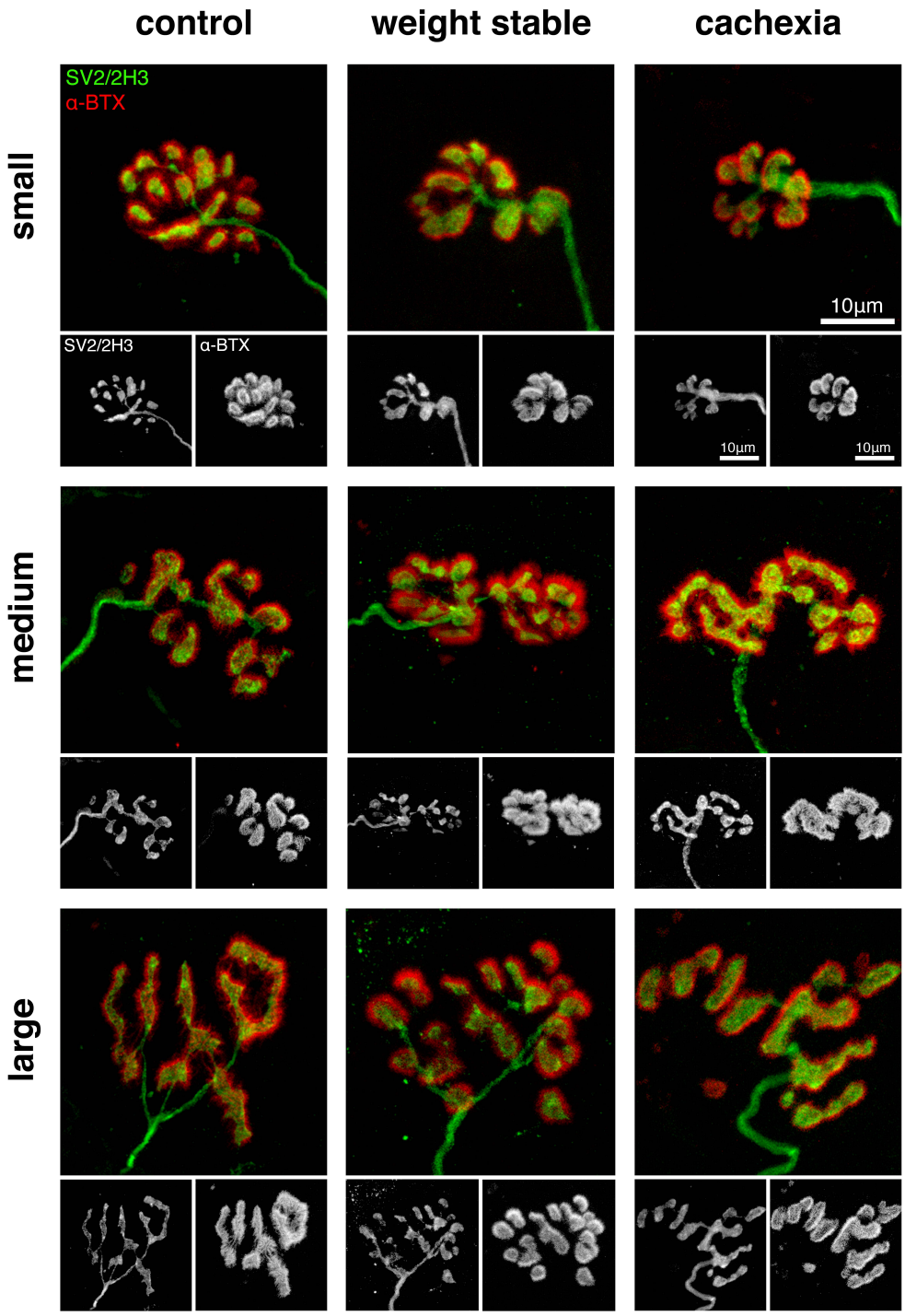


Figure 38 Conservation of NMJ morphology in cancer cachexia

Confocal micrographs of representative human NMJs from rectus abdominis in the three patient groups. Despite the heterogeneity in size and shape of individual NMJs, overall morphology was remarkably conserved across the three groups, with no evidence of NMJ pathology in either the cachexia or weight stable groups compared with controls. Axon and nerve terminals in green (2H3/SV2) and AChRs of the motor endplate in red (α-BTX). Scale bar = 10 μm.

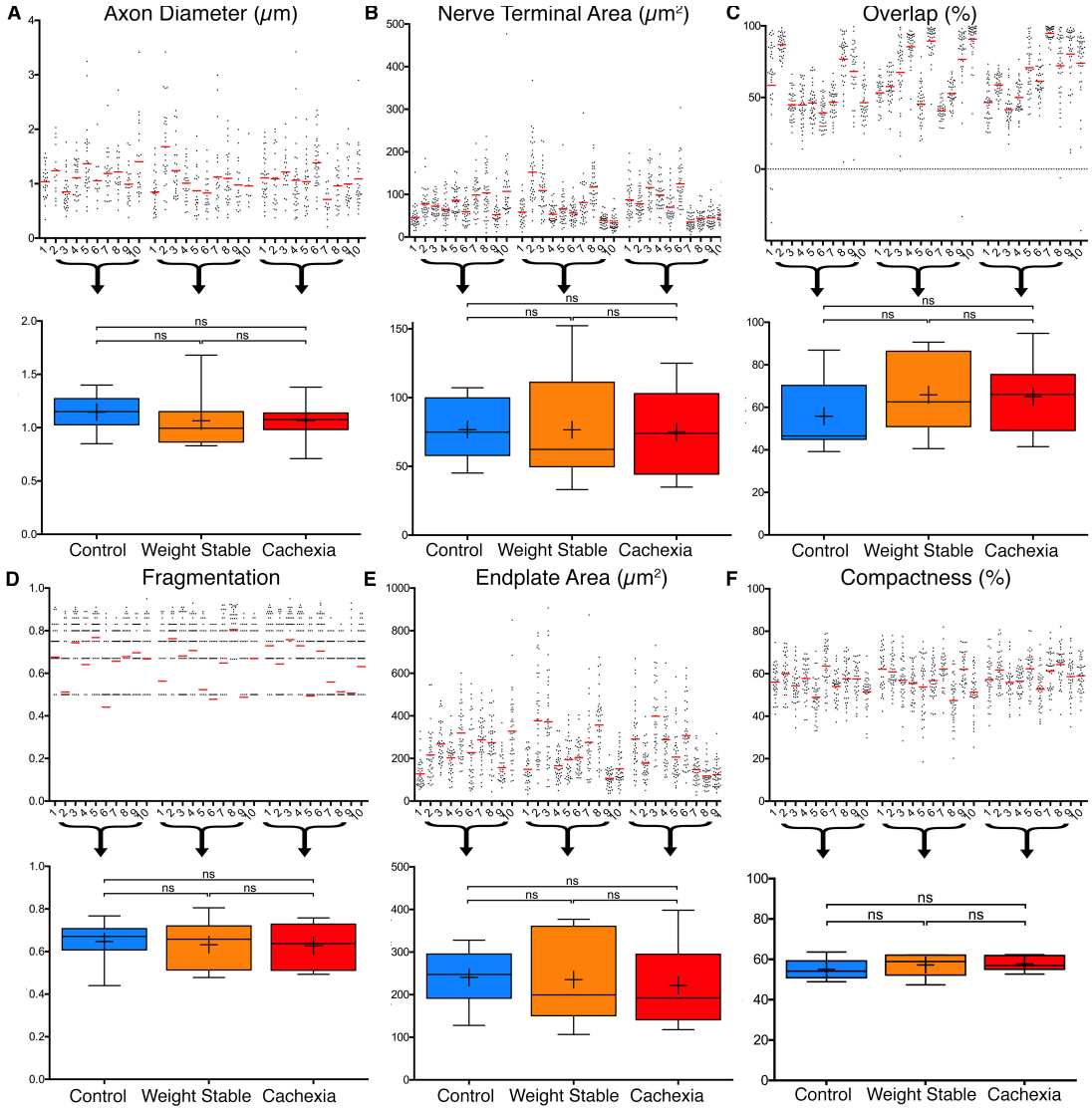


Figure 39 Structural integrity of the NMJ in cancer cachexia

Morphometric analysis using NMJ-morph did not reveal any significant differences in NMJ architecture between the three groups, with NMJ morphology being conserved in both cachexia and weight stable disease. Data presented as a pair of charts (scatterplot, above; box & whisker plot, below) for key NMJ variables, including measurements of pre- and post-synaptic architecture and axon diameter. Scatterplots depict the ~40 individual NMJs (data points) for the 10 patients (1 to 10) in each group; the mean NMJ value is given by the red line; the observed heterogeneity is a normal feature of human NMJ morphology. Box and whisker plots constructed using the mean patient data in each group (10 patients; control NMJs n = 387, weight stable NMJs n = 386, cachexia NMJs n = 392). The box contains the mean (+) and median (line) values for each NMJ variable and encloses the central 90% of the data; the whiskers represent the SEM. One-way ANOVA paired with a Tukey's post-hoc test (ns = not significant).

Although initial qualitative observations suggested an absence of gross pathology at the NMJ, it is possible that more subtle changes in NMJ morphology could have still been present in the cachectic patients. A comprehensive NMJ-morph analysis of all patient NMJs was therefore undertaken (Table 21). NMJs from rectus abdominis were initially compared with an existing database of human NMJs from several lower limb muscles (including extensor digitorum longus, peroneus longus, peroneus brevis and soleus to determine their likeness (or otherwise) to established human NMJ morphology in other muscle groups. NMJ-morph analysis revealed comparable NMJ morphology in rectus abdominis and the four lower limb muscles, in keeping with the gross appearances on initial inspection (Figure 40).

	Ctrl n = 10 387 NMJs	Weight Stable n = 10 387 NMJs	Cachexia n = 10 392 NMJs	F (DFn, DFd) or Kruskal Wallis statistic	P	Mean Difference Ctrl vs Weight Stable	Mean Difference Ctrl vs Cachexia	Mean Difference Weight Stable vs Cachexia
core variables								
pre-synaptic								
1) *Nerve Terminal Area (µm²)*	76.64 ± 6.89	76.62 ± 12.11	74.99 ± 10.01	F (2, 27) = 0.009	0.9909	0.011	1.646	1.635
2) *Nerve Terminal Perimeter (µm)*	143.62 ± 10.12	138.88 ± 14.25	134.93 ± 11.66	F (2, 27) = 0.128	0.88	4.741	8.682	3.941
3) *Number of Terminal Branches*	38.19 ± 3.47	36.88 ± 2.32	37.33 ± 2.50	0.008	0.9961	-0.300	0.000	0.300
4) *Number of Branch Points*	17.66 ± 3.26	17.12 ± 1.84	17.68 ± 1.93	F (2, 27) = 0.017	0.9831	0.540	-0.020	-0.559
5) *Total Length of Branches (µm)*	64.55 ± 6.33	62.14 ± 7.13	60.07 ± 5.98	F (2, 27) = 0.119	0.888	2.414	4.486	2.072
post-synaptic								
6) *AChR Area (µm²)*	130.03 ± 9.69	128.97 ± 17.62	124.75 ± 15.01	F (2, 27) = 0.037	0.9635	1.061	5.287	4.225
7) *AChR Perimeter (µm)*	150.90 ± 9.47	154.14 ± 14.61	153.77 ± 18.95	F (2, 27) = 0.014	0.9859	-3.238	-2.868	0.371
8) *Endplate Area (µm²)*	240.77 ± 21.01	235.29 ± 32.27	221.51 ± 29.81	F (2, 27) = 0.125	0.8833	5.483	19.27	13.79
9) *Endplate Perimeter (µm)*	77.82 ± 4.27	78.32 ± 6.54	77.29 ± 6.68	F (2, 27) = 0.007	0.9926	-0.497	0.528	1.025
10) *Endplate Diameter (µm)*	27.29 ± 1.32	28.79 ± 2.44	27.96 ± 2.25	F (2, 27) = 0.133	0.876	-1.502	-0.678	0.824
11) *Number of AChR Clusters*	4.16 ± 0.29	4.20 ± 0.41	4.29 ± 0.45	F (2, 27) = 0.031	0.9694	-0.049	-0.136	-0.086
derived variables								
pre-synaptic								
12) *Average Length of Branches (µm)*	1.91 ± 0.11	1.82 ± 0.15	1.79 ± 0.10	F (2, 27) = 0.29	0.7506	0.098	0.126	0.027
13) *Complexity*	4.35 ± 0.15	4.34 ± 0.11	4.37 ± 0.12	F (2, 27) = 0.007	0.993	0.009	-0.012	-0.021
post-synaptic								
14) *Average Area of AChR Clusters (µm²)*	42.29 ± 4.64	39.19 ± 4.05	38.49 ± 2.53	F (2, 27) = 0.278	0.7598	3.107	3.804	0.697
15) *Fragmentation*	0.65 ± 0.03	0.63 ± 0.04	0.63 ± 0.03	F (2, 27) = 0.107	0.8994	0.015	0.021	0.006
16) *Compactness (%)*	56.14 ± 1.33	56.88 ± 1.61	58.95 ± 1.11	F (2, 27) = 1.142	0.3341	-0.740	-2.812	-2.071
17) *Overlap (%)*	55.83 ± 5.11	65.90 ± 5.89	64.98 ± 5.23	F (2, 27) = 1.054	0.3624	-10.07	-9.148	0.919
18) *Area of Synaptic Contact (µm²)*	71.18 ± 7.59	81.81 ± 10.98	74.51 ± 4.41	F (2, 27) = 0.448	0.6433	-10.63	-3.332	7.295
associated nerve & muscle variables								

Table 21 NMJ Morph data. The mean values +/- SEM are listed for each patient group, representing the mean of the entire analysed NMJ data set. Statistical significance from a one-way ANOVA paired with a Tukey's post-hoc test. Statistical significance of average area of AChR clusters and fragmentation determined via Kruskal Wallis non-parametric test, followed by a Dunn's post-hoc test.

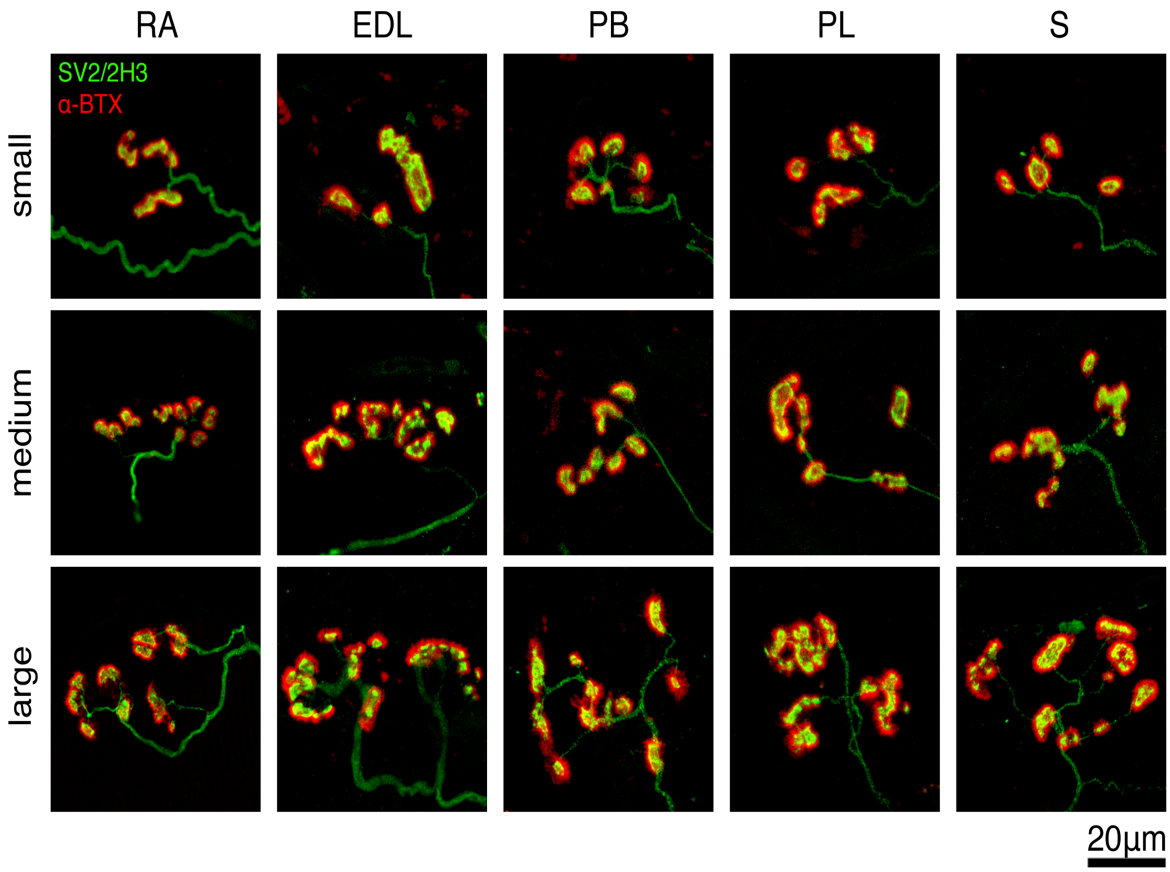


Figure 40 Comparative anatomy of rectus abdominis (RA), extensor digitorum longus (EDL), peroneus brevis (PB), peroneus longus (PL) and soleus (S) in humans

No major differences between the muscles were observed between lower limb muscles extensor digitorum longus (EDL, n = 10 patients), peroneus brevis (PB, n = 10 patients), peroneus longus (PL, n = 10 patients), soleus (S, n = 10 patients) and rectus abdominis (RA, n = 10 patients). The lower limb data of 10 patients from the previously published data set in Jones et al 2017 was chosen due to its completeness of pre- and post-synaptic parameters. Representative confocal images depicting the homogeneity in NMJ morphology across five different muscles. Heterogeneity in NMJ size is present in all five muscles. SV2/2H3 = synaptic vesicle 2 and neurofilament (presynapse, green); α-BTX = α-bungarotoxin (acetylecholine receptors, red). Scale bar = 20 µm.

Quantitative NMJ-morph analyses confirmed that there were no significant differences in any aspect of NMJ morphology in the rectus abdominis across the three patient groups. Crucially, there was no evidence of denervation (given by % overlap between the nerve terminals and motor endplate). The identification of displaced mononuclei in cachectic patients and C26 tumour bearing mice have previously been used to suggest the presence of denervation (50). However, detailed analyses of the NMJ confirmed that denervation is not a major feature of pathogenesis in cachectic patients.

Similarly, there was no evidence for increased NMJ fragmentation (breaking up of the motor endplate into discrete AChR islands) in either of the cancer groups compared with the control cases, a classical feature of NMJ pathology found in animal models of neurodegeneration (298–301). Animal models of cardiac cachexia have demonstrated disaggregation of AChRs in the soleus muscle of rats where heart failure was induced with monocrotaline (51). Again, detailed analyses of the NMJ confirmed that fragmentation of the NMJ is not a major feature of pathogenesis in cachectic patients.

Alongside analyses of denervation and NMJ fragmentation, NMJ-morph analysis confirmed no statistically-significant changes in any of the other morphological variables investigated (Figure 39; Table 21); similar axon diameters (~1um) were observed across all 3 groups with no evidence of axonal swelling or neurofilament accumulation), and no examples of

polyneuronal innervation were found (indicative of denervation/reinnervation processes).

5.5 Discussion

The complex pathogenesis involved in cachexia is not yet fully understood with theories about the involvement of the NMJ to date being largely based on animal models of sarcopenia (317–321). Since the consensus definition of cachexia focuses on the importance of muscle wasting, the NMJ could conceivably contribute through impaired transmission efficiency and impaired stability leading to denervation. Many morphological and electrophysiological changes have been observed at the NMJ in ageing animals including fragmentation, denervation, polyinnervation and axon sprouting. However whether these changes contribute to the pathogenesis of sarcopenia or cachexia still remains up for debate. In the present study the human NMJ was found to display a remarkable degree of structural stability in both cachectic and weight stable cancer patients, with no evidence of denervation or remodelling that has been demonstrated previously in animal models. These findings provide fundamental new insights into the role of the NMJ in cancer and cancer cachexia.

A well-studied and major morphological change of the rodent NMJ is that of fragmentation of the post synaptic AChR clusters (the division of the motor

endplate into discrete AChR islands). It is thought to coincide with muscle weakness and has the potential to be reversed by exercise (298). However, some studies have shown that neuromuscular transmission is increased in ageing mice due to the increase in quantal content (322,323). Aging human NMJs have been associated with an increase in axon diameter and area of nerve terminal which has previously been shown to be associated with quantal content (314). Similar axon diameters ($\approx$ 1μm) were observed across all 3 groups (with no evidence of axonal swelling or neurofilament accumulation) in this study implying no increase in quantal content. A study of human NMJs has also shown no evidence of fragmentation into the 10th decade of life suggesting that age related fragmentation is limited to rodents (46). It is important to note however that although older patients were used in this study muscle mass and force was not measured. Animal models of cardiac cachexia have demonstrated disaggregation of nAChRs in the soleus muscle of rats induced with heart failure by monocrotaline. Aggregates of nAChRs were distributed as continuous branches that were elongated, smooth and fluorescent forming continuous channels along the muscle fibre and presented a spot or island pattern (51). This suggested that the NMJs had undergone potential fragmentation and remodelling. Investigation of nAChR subunit gene expression showed upregulation of the alpha1 and epsilon subunits of nicotinic AChR in the heart failure group (51). The present study disagrees with these results demonstrating that there was no fragmentation of the NMJ in any of the

patient groups. This again may be a feature of cachexia that is limited to animal models.

Post mortem studies along with animal models have shown that sarcopenic loss of muscle fibres is linked to a loss of motor neurons associated with low muscle volume and contractility (321,324). In previous studies fast twitch fibres have demonstrated a greater reduction than slow twitch fibres in a sex-specific fashion (318). *Rectus abdominis* consists predominantly of type I (slow-oxidative) and 2A (fast oxidative-glycolytic) fibres (325), whereas type 2B (fast-glycolytic) fibres have been shown to be more susceptible to atrophy (326,327). Previous studies in rodents have shown correlations between fibre type switching and muscle atrophy in wasting diseases (318) and it is conceivable that the present observations may be confounded by such factors. Roles for displaced mononuclei in cachectic patients and C26 tumour bearing mice have been also been highlighted as a sign of denervation. Non-peripheral myonuclei have been observed upon denervation in the absence of molecular markers of muscle regeneration e.g. embryonic or fetal myosin. The number of nuclear clusters were also upregulated in cachexia alongside levels of neural cell adhesion molecule (NCAM); a marker of denervation suggesting this may be a underlying process in cachexia pathogenesis (50). Crucially in this study there was no evidence of denervation as evidenced by the percentage overlap between the nerve terminals and motor endplate.

Autophagy plays an important role in the regulation of the agrin-MuSK-Lrp4 signalling axis in stabilising the NMJ. Several animal models and studies of human gene expression have demonstrated that activity dependent regulation of autophagy and the agrin-MuSK-Lrp4 signalling axis are of great importance in stabilising the NMJ (328,329). Based on these insights, agrin-derived fragments are currently being evaluated as biomarkers for age-related muscle wasting. Investigation of levels of C-terminal Agrin (a marker of muscle wasting caused by degeneration of NMJs) in patients with heart failure were elevated in the majority of patients who had DEXA proven muscle wasting suggesting a link between NMJ degeneration and cachexia (310). Remodelling of the NMJ is thought to precede sarcopenia and be due to a loss of proteins including lamin-α4, β2 or integrin-α3 (317). Altered expression of Lamin-α4 in particular has been noted in wild type NMJs prior to an age related decline in transmission (330,331). Lamin-α4 knock out mice have shown altered measures of NMJ transmission but no change in muscle wasting suggesting a role for loss of function only (330). Proteomic profiling in humans has revealed distinct distribution of active zone proteins (46) but functional measurements of the NMJ were out-with the scope of this study and provide the premise for future investigations.

5.5.1 Limitations

It should be noted that the cachectic patients recruited into the current study represent the more extreme end of the diagnostic definition criteria, having

both low CT muscularity together with weight loss. They were found to have a significantly reduced mean muscle fibre diameter consistent with previous studies (312). This definition was deliberately chosen, rather than weight loss alone, to ensure the presence of histological wasting. However, this study only enrolled patients who were eligible for curative surgery. It is not possible, therefore, to draw conclusions concerning a possible role for the NMJ in the development of refractory cachexia or active muscle wasting.

The groups recruited were not sex matched as this was an exploratory study and not designed to detect differences in sexual dimorphism. This may explain the higher muscle fibre diameter seen in the control group as they contained only male patients. There was still however a significant difference between the weight stable and cachectic groups which both contained female patients.

All patients received intravenous atracurium besilate during anaesthesia, which competitively displaces acetylcholine from its receptor sites. Its half-life is 17-21 minutes, but whether it has longer-lasting effects on the form or function of the NMJ is not known (332). Importantly, therefore, samples from the current study were compared with lower limb samples from patients who had received spinal anaesthesia only. No differences were observed between these groups, suggesting that the choice of anaesthetic was unlikely to have had any significant impact on the morphological variables of the NMJ measured here.

It is also important to note that the majority of patients in this study may have had some degree of age related sarcopenia that may co-exist with cancer specific loss of muscle and that the diagnostic criteria may not necessarily separate one from the other. The study only captured patients who were eligible for curative surgery and therefore it cannot be said if the NMJ plays a role in the development of refractory cachexia or active muscle wasting. The control patients used were a mixture of patients undergoing aortic aneurysm repair and donor nephrectomy. Whilst the aortic patients do not necessarily represent a completely healthy cohort, they do represent a group that are free of cancer. Previous CT based muscularity studies have highlighted varying measures of muscularity between different patient groups and therefore demonstrates the importance of their inclusion in studies of muscle wasting (333).

5.6 Conclusion

In summary, the NMJ retains full structural stability in both cachectic and weight stable cancer patients. This suggests that denervation of skeletal muscle is not a major driver of disease pathogenesis. The absence of NMJ pathology in human cancer is in contrast to observations noted in animal models, and supports the hypothesis that intrinsic changes within skeletal muscle, independent of any changes in motor neurons, represent the primary locus of pathology in cachexia. Since the NMJ remains intact in patients with

cancer cachexia, promotion of muscle hypertrophy using neural stimulation should remain a viable therapeutic intervention for future clinical trials.

CHAPTER 6

ADIPOSE DEPOT GENE EXPRESSION IN CANCER CACHEXIA

6.1 Chapter overview

The fifth chapter focussed on understanding a possible mechanism of muscle wasting. The second forth however, had highlighted the need to better understand differences in adipose depot wasting. During cachexia, different adipose depots demonstrate differential wasting rates. Studies from animal models have suggested adipose tissue may be a key driver of muscle wasting through fat-muscle crosstalk but human studies in this area are lacking.

6.1.1 Objectives

This chapter aimed to perform global gene expression profiling of visceral (VAT) and subcutaneous (SAT) adipose tissue from weight stable and cachectic cancer patients and healthy controls.

6.1.2 Methods

Cachexia was defined as >2% weight loss plus low CT-muscularity. Biopsies of SAT and VAT were taken from patients undergoing resection for oesophago-gastric cancer, and healthy controls (donor nephrectomy) (n=16 and 8 respectively). RNA was isolated and reverse transcribed. cDNA was hybridised to the Affymetrix Clariom S Microarray and data was analysed using R/Bioconductor. Differential expression of genes was assessed using empirical Bayes and moderated-t-statistic approaches. Category enrichment analysis was used with a tissue-specific background to examine the biological context of differentially expressed genes. Selected differentially regulated genes were

validated by qPCR. ELISA for Intelectin-1 was performed on VAT samples for the corresponding patients. The current cohort plus 12 additional patients from each group also had plasma Intelectin-1 ELISA carried out.

6.1.3 Results

In VAT versus SAT comparisons there were 2101, 1722 and 1659 significantly regulated genes in the cachectic, weight stable and control groups respectively. There were 2200 significantly regulated genes from VAT in cachectic patients compared to controls and 1253 significantly regulated genes from VAT in weight stable cancer patients compared to controls. The gene showing the largest difference in expression was Intelectin-1 (Omentin-1) (FDR corrected p=0.0001); a novel adipocytokine associated with weight loss in other groups. Genes involving inflammation were enriched in cancer and control VAT versus SAT though different groups of genes contributed. Energy metabolism and fat browning genesets were downregulated in cancer VAT as were key fat browning genes (e.g. UCP1).

6.1.4 Conclusion

SAT and VAT have unique gene expression signatures. VAT is metabolically active in cancer, and maybe a target for therapeutic manipulation. VAT may play a fundamental role in cachexia, but the downregulation of energy metabolism genes implies a limited role for fat browning in human cachectic patients, in contrast to pre-clinical models.

6.2 Introduction

Although the consensus definition of cancer cachexia (28) emphasises loss of lean tissue (particularly skeletal muscle) as a key identifier in the diagnosis of the condition, the loss of adipose tissue in cancer cachexia is increasingly thought to play an important role (335). A better understanding of the physiological effects of cancer on adipose tissue would open new therapeutic avenues for treatment resulting in improved outcomes.

In cancer, the loss of adipose tissue is driven by lipolysis (338) rather than adipocyte apoptosis or necrosis. Mediators of lipolysis in cancer cachexia are largely unknown. In the past, increased mRNA expression of zinc-α2-glycoprotein (ZAG), a proposed lipid mobilising factor, was identified in fat samples from cachectic patients; however, serum ZAG levels were unchanged from controls (339,340). Microarray analysis has revealed that changes in the transcriptome of subcutaneous fat in cachexia are opposite to those seen in obesity, underlining the importance of lipolysis (108). Visceral adipose tissue is thought to be lost more rapidly than subcutaneous adipose tissue during cachexia, suggesting differential adipose depot-dependent responses to the wasting process though this has not been consistently demonstrated in humans (341). Bioinformatic analysis of mRNA expression in visceral (omental) and subcutaneous adipose depots in non-cachectic, obese endometrial cancer patients demonstrated nineteen shared biological pathways, eighteen of which were regulated in opposite directions between

the fat depots (342). Recently, focus has concentrated on the concept of “fat-muscle crosstalk” in cancer cachexia (335). Notably, genetic ablation of lipolytic pathways in adipocytes protects against muscle mass loss in animal models of cancer cachexia (159). Inhibition of lipolysis through genetic ablation of adipose triglyceride lipase (Atgl) or hormone sensitive lipase (HSL) was shown to reduce muscle wasting (159). These data support the loss of visceral fat driven by lipolytic mechanisms as an early event in cancer cachexia with a potential effect on skeletal muscle.

Global gene expression profiling is an effective method to examine regulatory pathways in patho-physiological contexts (343). In previous studies, microarray analysis of skeletal muscle biopsies taken at the time of resectional surgery for UGI cancer revealed an 83-gene signature that was able to identify patients with >5% weight loss (344). In further studies, a transcriptomic comparison of sequential muscle biopsies (at surgery and 8 months post-surgery) from UGI cancer patients revealed 1,868 regulated genes associated with cancer and weight loss (345). Contrary to expectation, the vast majority (94%) of genes were downregulated. Category analysis of the differentially expressed genes showed that both anabolic and catabolic process gene expression was suppressed in cancer. Furthermore, there was a lack of substantive overlap with transcriptomic signatures from endurance training, strength training, ageing, or simple dieting. Highly enriched categories

included lipid oxidation, fatty acid metabolism, and peroxisome pathways, implying a role for fat-muscle crosstalk in cancer cachexia (346).

In the current study the global transcriptome of VAT and SAT depots in patients with confirmed cachexia was examined according to the diagnostic consensus definition (>2% weight loss and low muscularity on CT; n=8) compared with weight stable cancer patients (n=8), as well as healthy controls undergoing donor nephrectomy (n=8). These data provide the first examination of global gene expression in these two fat depots in cancer cachexia. The data provide a basis for hypothesis generation and exploring treatments.

6.3 Methods

6.3.1 Patient recruitment

UGI cancer and donor nephrectomy patients were recruited as previously described. Subcutaneous and visceral adipose tissue biopsies were taken from all patients as well as fasting venous blood samples. CT body composition analysis was performed. Patients were divided into three groups: cancer cachexia (CC), weight stable cancer (CWS) and control (Ctrl). Cancer patients were defined as being cachectic based on the consensus definition (>2% weight loss and low CT muscularity). Patient demographics are described in terms of median (interquartile range). Kruskal Wallis test was used to compare groups.

6.3.2 RNA extraction of fat

6.3.2.1 Cutting of fat

Fat that had been stored at -80°C was placed in dry ice and transferred to a piece of aluminium foil for cutting. A surgical blade and forceps were used to cut the fat into the desired sections. The forceps and blades were cleansed in vircon after each sample was taken to remove macroscopically visible tissue. Samples that had not been thawed were placed back in their storage tube and into the -80°C freezer thereafter.

Approximately 0.5cm of frozen fat was excised from the original biopsy sample and homogenised with 500µl of TRIzol. This step was performed as quickly as possible to ensure the tissue did not thaw. The lysate was centrifuged at full speed for three minutes and the supernatant removed by pipetting. It was then transferred to a new microcentrifuge tube. One volume of 70% ethanol was added to the cleared lysate and mixed immediately by pipetting. 700µl of the sample was transferred to an RNeasy spin column placed in a 2ml collection tube. This was centrifuged for 15s at >8000 x g (>10,000 rpm) and the flow-through was discarded. 700µl Buffer RW1 was added to the RNeasy spin column and centrifuged for 15 seconds at > 8000 x g (>10,000 rpm) to wash the spin column membrane. The flow-through was discarded. 500µl of Buffer RPE was added to the RNeasy spin column and centrifuged for 15 seconds at >8000 x g (>10,000 rpm) to wash the spin column membrane. Ethanol was added to the Buffer RPE before use. The flow-through was discarded. A further

500µl of Buffer RPE was added to the RNeasy spin column and centrifuged for 2 minutes at >8000 x g (>10,000 rpm) to wash the spin column membrane. The RNeasy spin column was placed in a new 2 ml collection tube and the old collection tube containing the flow-through was discarded. This was centrifuged at full speed for one minute. The RNeasy spin column was placed in a new 1.5ml collection tube. 30µl of RNase-free water was directly added to the spin column membrane and centrifuged for one minute at >8000 x g (>10,000 rpm) to elute the RNA. The quality of the RNA was then tested on a nanodrop.

6.3.2.2 Preparation of RNA with poly-A controls and synthesis of first strand cDNA

2 µl of serially diluted Poly-A RNA control was added to 100ng of RNA. Nuclease free water was added as required to make the total volume 5 µl. On ice the first strand master mix was prepared. The volume required for one reaction was 4 µl of first strand buffer and 1 µl of first strand enzyme. 5 µl of master mix was added to 5 µl of total RNA/Poly-A control mixture. This was incubated for 60 minutes at 25^{o}C, then for 60 minutes at 42^{o}C in a thermal cycler using the “first strand cDNA synthesis” program.

6.3.2.3 Synthesis of second strand cDNA

On ice the second strand master mix was prepared. The volume required for one reaction was 18 µl of second strand buffer and 2 µl of second strand

enzyme. 20 µl of second strand master mix was added to each (10 µl) first strand cDNA sample. This was incubated for 60 minutes at 16°C, then for 10 minutes at 65°C in a thermal cycler using the “second strand cDNA synthesis” program.

6.3.3.4 Synthesis of cRNA by in vitro transcription

The second strand cDNA samples were transferred to room temperature whilst the IVT master mix was prepared. The IVT master mix was also prepared at room temperature. The volume for one reaction was 24 µl of IVT buffer and 6 µl of IVT enzyme, giving a total volume of 30 µl per reaction. 30 µl of IVT master mix was transferred to each (30 µl) of second strand cDNA sample. The IVT reaction was incubated for 16 hours at 40°C in a thermal cycler using the “in vitro transcription cRNA synthesis” program.

6.3.3.5 Purification of cRNA

Nuclease-free water was preheated to 65°C. Purification beads were mixed before use at room temperature. 80% ethanol wash solution was prepared. The purification beads container was mixed to resuspend the magnetic particles. 100 µl of the magnetic beads were added to each (60 µl) cRNA sample. These were mixed by pipetting up and down and transferred to a well of a U-bottom plate. They were further mixed by pipetting up and down and the incubated at room temperature for 10 minutes. The plate was moved to a magnetic stand for 5 minutes to capture the magnetic beads. Whilst still on the

magnetic stand the supernatant was carefully aspirated and discarded without disturbing the magnetic beads. 200 µl of 80% ethanol was solution was added to each well and incubated for 30 seconds. The ethanol was then slowly aspirated without disturbing the magnetic beads. This wash was repeated a total of 3 times. The final wash solution was completely removed. The magnetic beads were air dryed on the magnetic stand for 5 minutes until no liquid was visible. The plate was removed from the magnetic stand. 27 µl of the preheated (65^{o}C) nuclease-free water was added to each sample and incubated for 1 minute. This was mixed well by pipetting up and down 10 times. The plate was moved to the magnetic stand for approximately 5 minutes to capture the magnetic beads. The supernatant which contains the eluted cRNA to a nuclease free tube. The purified cRNA was placed on ice and the cRNA yield was assessed using a NanoDrop.

6.3.3.6 Synthesis of 2nd cycle ss-cDNA

On ice 15 µg of cRNA was prepared in a volume of 24 µl nuclease free water. 4 µl of 2nd cycle primers were added to each (24 µl) cRNA sample. This was incubated at 70^{o}C for 5 minutes, 25^{o}C for 5 minutes and 4^{o}C for 2 minutes in a thermal cycler using the “2nd cycle primers cRNA annealing” program. The cRNA/2nd cycle primers were then placed on ice and the 2nd cycle ss-cDNA master mix was prepared. This included 8 µl of 2nd cycle ss-cDNA buffer and 4 µl of 2nd cycle ss-cDNA enzyme giving a total volume of 12 µl for one reaction. 12 µl of the 2nd cycle ss-cDNA master mix was added to each (28 µl)

cRNA/2nd-cycle primers sample. This was incubated for 10 minutes at 25°C, 90 minutes at 42°C and then 10 minutes at 70°C in a thermal cycler using the "2nd cycle ss-cDNA synthesis" program.

6.3.3.7 Hydrolysing RNA using RNase H

On ice 4 µl of RNase H was added to each (40 µl) 2nd cycle ss-cDNA sample. This was mixed thoroughly, followed by a quick spin. This was incubated at 37°C for 45 minutes in a thermal cycler using the "RNA hydrolysis" program. The hydrolysed 2nd cycle ss-cDNA samples were placed on ice and 11 µl of nuclease free water was added to each (44 µl) hydrolysed 2nd cycle ss-cDNA sample. This was mixed thoroughly and immediately frozen at -20°C.

6.3.3.8 Purification of 2nd cycle ss-cDNA

Nuclease free water was pre-heated to 65°C. Fresh dilutions of 80% ethanol were prepared. The purification bead container was mixed to resuspend the magnetic particles. 100 µl of the magnetic beads were added to each (55 µl) hydrolysed ss-cDNA sample. These were mixed by pipetting up and down, and transferred to a well of a U-bottom plate. 150 µl of 100% ethanol were added to each sample. These were mixed well by pipetting up and down 10 times and incubated for 20 minutes. The plate was moved to a magnetic stand and incubated for 5 minutes to capture the magnetic beads. The supernatant was carefully aspirated and discarded without disturbing the beads. Whilst still on the magnetic stand 200 µl 80% ethanol wash solution was added to each well

and incubated for 30 seconds. The ethanol was slowly aspirated and discarded without disturbing the magnetic beads. This was repeated for a total of 3 washes. The final wash solution was completely removed and the beads were left to air dry on the magnetic stand for 5 minutes until no liquid was visible. The plate was removed from the magnetic stand. 30 µl of the preheated (65^{o}C) nuclease free water was added to each sample and incubated for 1 minute. Samples were mixed well by pipetting up and down 10 times. The plate was moved to the magnetic stand for 5 minutes to capture the magnetic beads. The supernatant which contained the eluted ss-cDNA was transferred to a nuclease free tube. The purified ss-cDNA was placed on ice and the yield was quantified using a NanoDrop.

6.3.3.9 Fragmentation and labelling of ss-cDNA

On ice 5.5µg of ss-cDNA was prepared in a volume of 31.2µl with nuclease free water. On ice the fragmentation and labelling master mix was prepared. This contained 10µl of nuclease free water, 4.8 µl 10X cDNA fragmentation buffer, 1µl of UDG, 10 U/µl and 1µl of APE 1, 1000U/µl giving a total volume of 16.8µl for each reaction. 16.8µl of the fragmentation master mix was added to each (31.2µl) 5.5µg ss-cDNA sample. This was incubated for 60 minutes at 37^{o}C, then 2 minutes at 93^{o}C in a thermal cycler using the “fragmentation” program. On ice 45µl of the fragmented ss-cDNA was added to an individual well. The labelling master mix was prepared on ice. This contained 12µl of 5X TdT buffer, 1µl of DNA labelling reagent, 5mM and 2µl of TdT, 30 U/µl giving

a total volume of 15µl for each reaction. 15µl of the labelling master mix was added to each (45µl) fragmented ss-cDNA sample. This was incubated for 60 minutes at 37^{o}C, then for 10 minutes at 70^{o}C in a thermal cycler using the "labelling" program.

6.3.3.10 WT cartridge array hybridization

The 20X hybridisation controls were heated for 5 minutes at 65^{o}C in a thermal cycler using the "hybridisation control" program. The hybridisation master mix was prepared at room temperature.

The hybridisation master mix was added to individual tubes containing the biotin labelled ss-cDNA sample to prepare the hybridisation cocktail.

The hybridisation cocktail was incubated at 5 minutes for 95^{o}C, then 5 minutes at 45^{o}C in a thermal cycler using the "hybridisation cocktail" program. 80µl was loaded onto the array and hybridised with rotation at 60 rpm for 16 hours at 45^{o}C (figure 41).

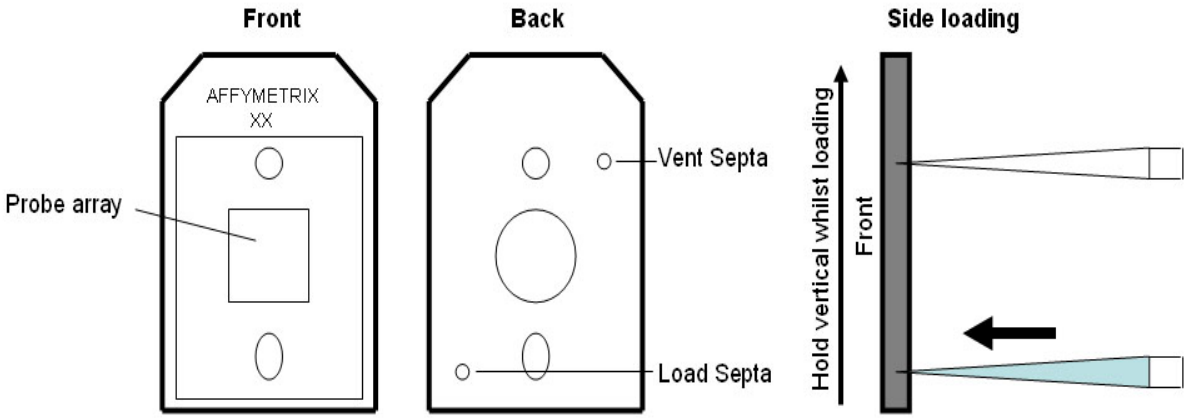


Figure 41 Loading of hybridisation cocktail onto arrays

Vent septa were opened and the hybridisation cocktail was pipetted into the load septa. Care was taken not to have any visible air bubbles in the arrays.

6.3.3.11 Wash and staining

Arrays were removed from the oven and the hybridisation mix extracted on ice. Each array was completely filled with wash buffer at room temperature. Vials containing 600 µl of stain cocktails 1 and 2 and 800 µl of array holding buffer were placed into sample holders and the fluidics protocol was commenced (figure 42).

Figure 42 Fluidics protocol

After washing and staining images were captured and cel files generated using the GeneChip Scanner 3000 (Thermofisher UK). The cel files can be accessed at the Gene Expression Omnibus using the ID GSE131835. A summary of this process is shown in figure 43.

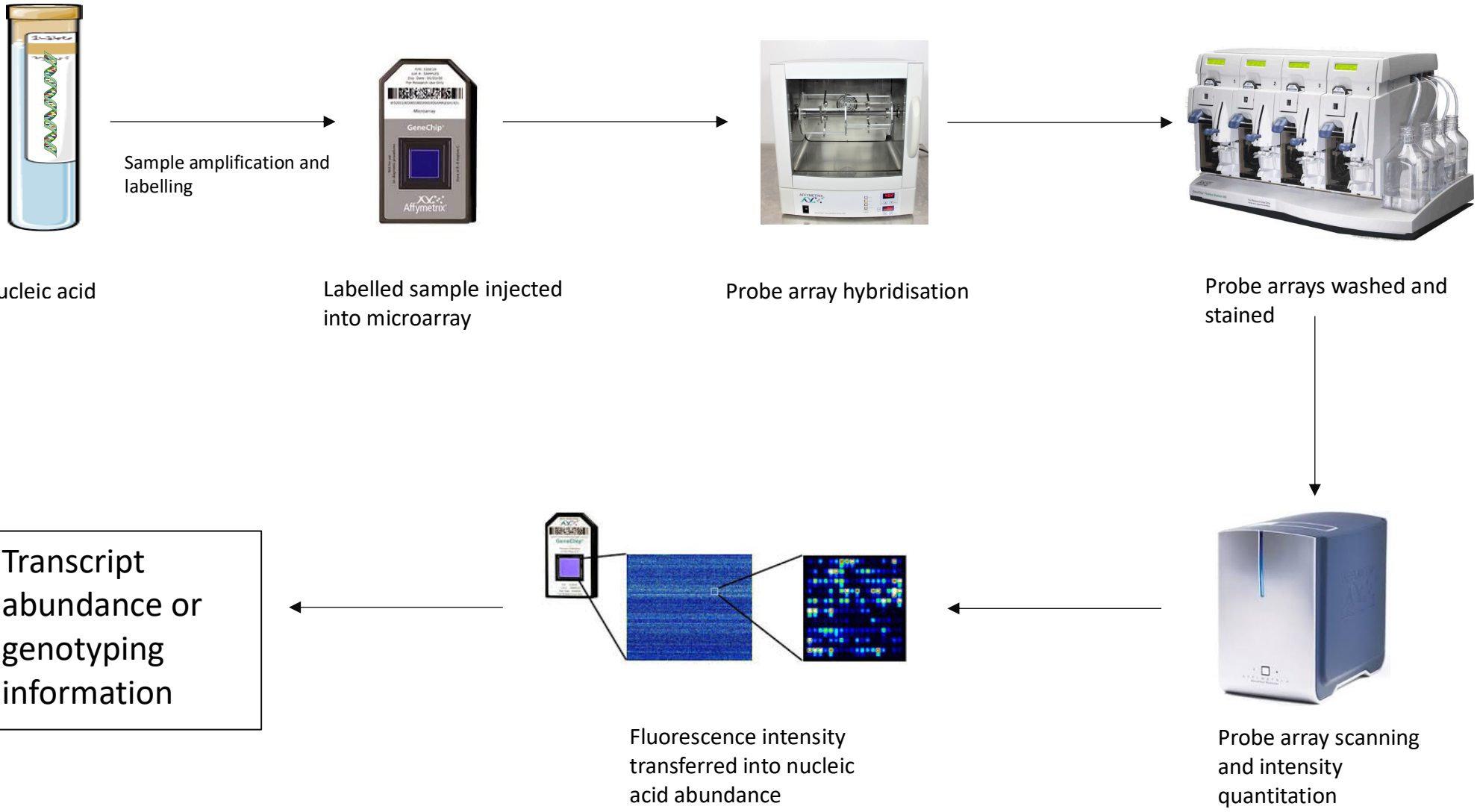


Figure 43 Summary of transcriptomic analysis methods

6.3.3 QRT-PCR

Total RNA was extracted and cDNA was prepared as described above. First strand cDNA synthesis was carried out using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, cat no. 4368814) with 500ng total RNA input for each reaction. Quantitative PCR (qPCR) was carried out using Luminaris Hi Green Low ROX qPCR Master Mix (Thermofisher UK, Cat no. K0974) 5ul, 0.3ul forward and reverse primer at 10uM each and 3.4ul ddH_2O. Primers were selected from the Primer Bank resource (347) except for ACTB and GAPDH. These latter primers were designed to span exons using the Primer-BLAST resource (348). Primer sequences are detailed in table 22. We selected primers to cover genes involved in inflammation (ACVR2A1, IL6, IL18), adipogenesis (LEPR, STAT5A, PDCD4), adipose browning (UCP1, PPARGC1A, PRDM16) as well as genes which demonstrated a substantial fold change by microarray data (ITLN1, ALOX15, PPARA). We also profiled 5 genes to use as reference values in PCR data normalisation as recommended by the MIQE guidelines (349). PCR reactions were carried out on the Lightcycler 480 Instrument with the following protocol; 50^{o}C, 2mins, 95^{o}C 10mins and 40 cycles of 95^{o}C 15s and 60^{o}C 60s before a final melting curve step using triplicate reactions per sample in 384 well plates. Analysis of qPCR data was carried out using the NormqPCR package in R/Bioconductor (350). The genorm method (351) was used to identify the four most stable control genes from ACTB, GAPDH, YWHAZ, POLR2A and PSMB2 and the geometric

mean of these was used to normalise the cycle threshold values for the remaining genes.

Gene	Category	Forward primer	Reverse primer	Primer Bank ID
ACVR2A	Inflammation	GTTTGCCGTCTTTCTTATCTCCT	GTCACCATAACACGGTTCAACA	65508448c1
IL6	Inflammation	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG	224831235c1
IL18	Inflammation	TCTTCATTGACCAAGGAAATCGG	TCCGGGGTGCATTATCTCTAC	342349317c1
LEPR	Adipogenesis	ACCTCTGGTTCCCCAAAAAGG	TTGGCACAGGCACAAGACAT	310923190c1
STAT5A	Adipogenesis	GCAGAGTCCGTGACAGAGG	CCACAGGTAGGGACAGAGTCT	221316717c1
PDCD4	Adipogenesis	GCAAAAAGGCGACTAAGGAAAAA	TAAGGGCGTCACTCCCACT	313760536c1
UCP1	Browning	CAATCACCGCTGTGGTAAAAAC	GTAGAGGCCGATCCTGAGAGA	194733736c2
PPARGC1A	Browning	TCTGAGTCTGTATGGAGTGACAT	CCAAGTCGTTCACATCTAGTTCA	116284374c1
PRDM16	Browning	CGAGGCCCCTGTCTACATTC	GCTCCCATCCGAAGTCTGTC	289547570c1
ITLN1	Microarray	AGTGTTGGACTGACAACGGC	TACATCCGGTGACCCTCATTC	31542985c2
ALOX15	Microarray	TGGAAGGACGGGTTAATTCTGA	GCGAAACCTCAAAGTCAACTCT	40316936c2
PPARA	Microarray	TTCGCAATCCATCGGCGAG	CCACAGGATAAGTCACCGAGG	61744436c2
ACTB	Reference	AATGTGGCCGAGGACTTTGATTGC	AGGATGGCAAGGGACTTCCTGTAA	NA
POLR2A	Reference	CCTGAGGGTGACGAGGATCT	GTGCTTCCATTCCGCATACAG	306482654c2
PSMB2	Reference	ATCCTCGACCGATACTACACAC	GAACACTGAAGGTTGGCAGAT	315139005c1
YWHAZ	Reference	TGTAGGAGCCCGTAGGTCATC	GTGAAGCATTGGGGATCAAGA	208973243c2
GAPDH	Reference	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG	NA

Table 22 Primer sequences used in qPCR validation

6.3.4 ELISA

Intelectin-1 concentration was assessed in the VAT and plasma using enzyme-linked immunosorbent assay (ELISA) (Amsbio EH0564); sensitivity <46.875pg/ml; reference range: 78.125-5000pg/ml). A further 36 patients were recruited to increase the total of plasma samples to 20 for each group. Pre-experiment dilution testing was performed on one plasma sample from each group. Based on standard curves produced, a medium target protein concentration was chosen (5000-50000pg/ml) 1:10 dilution. Reagents were prepared according to manufacturers instructions (Amsbio). Plates were washed twice with wash buffer before samples were added. Intra and interassay coefficients of variation were <8% and <10% respectively.

0.1ml of 500pg/ml,2500pg/ml, 1250pg/ml, 625pg/ml, 312.5pg/ml, 156.25pg/ml, 78.125pg/ml, standard solutions were aliquoted into the standard wells. 0.1 ml of Sample / Standard dilution buffer was added into the control (zero) well. 0.1ml of sample was added into the test sample wells. The plate was sealed with a cover and incubated at 37 °C for 90 min. The cover was removed and the plate content discarded making sure the wells did not completely dry at any time. 0.1 ml of Biotin-detection antibody working solution was added into the above wells (standard, test sample & zero wells). The solution was added at the bottom of each well without touching the side wall. The plate was sealed with a cover and incubated at 37°C for 60 min. The cover was removed, and the plate washed 3 times with Wash buffer. 0.1 ml of SABC working solution

VAT ELISA was performed by Gillian Dreczkowski, University of Stirling

was added into each well, the plate covered and incubated at 37°C for 30 min. The cover was removed and the plate washed 5 times with Wash buffer, each time the wash buffer was left in the wells for 1-2 min. 90 µl of TMB substrate was added into each well, the plate covered and incubated at 37°C in the dark for 15 minutes. Shades of blue can be seen in the first 3-4 wells (with most concentrated ITLN1 standard solutions), the other wells show no obvious color. 50 µl of Stop solution was added into each well and mixed thoroughly. The color changes into yellow immediately. The O.D. absorbance was read at 450 nm in a microplate reader immediately after adding the stop solution.
The standard curve was plotted as the relative O.D.450 of each standard solution (Y) vs. the respective concentration of the standard solution (X). The ITLN1 concentration of the samples was then interpolated from the standard curve.

Tissue ELISA was carried out on homogenised VAT tissue. Adipose tissue was homogenised in RIPA buffer with added protease inhibitors (ThermoFisher, UK). Tissue aliquots were added to 1.5ml PowerBead tubes with 1.4mm ceramic beads (Qiagen cat no 13113) and shaken at 5000rpm for 20s with 10s rest on ice 3 times using the MagNALyser instrument (Roche, UK). The homogenate was centrifuged at 5000rpm for 15minutes at 4^{o}C and the aqueous phase beneath the lipid cake and above the cell pellet was removed to a separate tube. Aliquots were diluted 1:20 in PBS and protein content quantified

using the Qubit 4 Protein Assay (ThermoFisher). The same ELISA kit was used for VAT and plasma (Amsbio EH0564).

6.3.5 Statistical analysis

Microarray were analysed using R/Bioconductor using an updated chip definition file (CDF) from the Brainarray resource (352) based on Ensembl gene definitions (ENSG version 22). Raw microarray data were normalised and filtered using the SCAN-UPC method (353). Expression intensity and UPC filters were set at 0 and 0.5 respectively. Use of these filters suggested 49% of the genes with probes on the microarray CDF were not expressed in adipose tissue. This is in line with my collaborators previous experience using GeneChip microarrays. Filtering also improved the clustering of samples by principle components analysis (PCA). Changes in differential expression were examined using empirical Bayes and a moderated t statistic implemented in the limma package (354) with subject identifier as a blocking factor in linear modelling to account for the repeated measures structure (see section Additive Models and Blocking in the limma manual). Category enrichment analysis was carried out using the camera algorithm (355) and the Broad Institute curated Hallmark Genesets collection (356). A FDR <5% was taken to indicate significant enrichment of a category.

The qPCR data were analysed using the R/Bioconductor NormqPCR package (350). The expression data was modelled with a mixed effects linear model

Assistance with statistical analysis was given by Dr Iain Gallagher, University of Stirling

using the lme4 R package (357) with subject group, fat depot and the interaction between these as fixed factors and subject identifier as a random factor to account for pairing. Post-hoc testing of interaction or main effects was carried out using the emmeans (358) package.

ITLN1 concentrations were calculated from raw ELISA data in R using the nCal package (359). After subtraction of blank values as recommended by the kit manufacturer a 5-PL model was fit to the data and concentrations back calculated. Statistical analysis was carried out on log transformed data. Tissue ELISA data were analysed using a linear model with log ITLN1 concentration modelled by group. For the plasma ELISA data the log ITLN1 concentrations from assays 1 & 2 were combined and modelled with a mixed effects model using the lme4 package (357) with assay as a random factor. Post hoc testing was carried out using the emmeans package (358).

6.4 Results

6.4.1 Patient characteristics

Twenty-four patients were included in the study with 8 subjects in each of the control, CWS and CC groups. Patient demographics and anthropometric details are shown in table 23. Median weight loss was 12.5% in the cachectic group (IQR 6.87-15.5, $p<0.001$). Control patients were younger (median 51 years compared to 60 in the CWS group and 70 in the CC group, $p=0.002$).

Skeletal muscle index was significantly different between the groups (p=0.002) (Table 23).

Group (n=8)	Control	Cachexia	Weight stable	P value
Male:Female	4:4	5:3	6:2	/
Age (years)	51 (48-55)	70 (64-77)	60 (57-64)	0.002
BMI (kg/m^2)	25.5 (23.75-28.25)	24 (20.25-29.25)	31.5 (23-40)	0.227
%weight loss	0	12.5 (6.87-15.5)	0	<0.001
SMI cm^2/m^2	48.30 (11.05-43.06)	39.99 (38.24-41.13)	55.52 (48.40-64.22)	0.002
VATI cm^2/m^2	20.97 (11.05-43.06)	23.3 (5.82-38.76)	55.52 (48.40-64.22)	0.196
SATI cm^2/m^2	50.32 (29.46-63.43)	50.75 (20.15-98.77)	70.35 (34.04-120.67)	0.605
VAT CSA cm^2	61.27 (29.77-122.65)	68.97 (18.16-102.12)	179.18 (80.43-251.94)	0.018
SAT CSA cm^2	159.29 (129.72-211.40)	154.10 (60.44-271.01)	214.42 (117.01-297.11)	0.013

Table 23 Demographic and selected clinical data for adipose study subjects

Median (Interquartile range) values are presented

6.4.2 Microarray analysis

Filtering the microarray data as recommended by the SCAN-UPC authors (353) led to the identification of 8679 probesets (approximately 49%) as unlikely to be expressed in adipose tissue. These were removed from the data set prior to further analysis to improve statistical power. Principle components analysis indicated a substantive difference in visceral versus subcutaneous transcriptome profiles in the presence of cancer (Figure 44) so we first examined mRNA expression differences between the subcutaneous and visceral depots within each group using a 5% false discovery rate (FDR) cut off for significance.

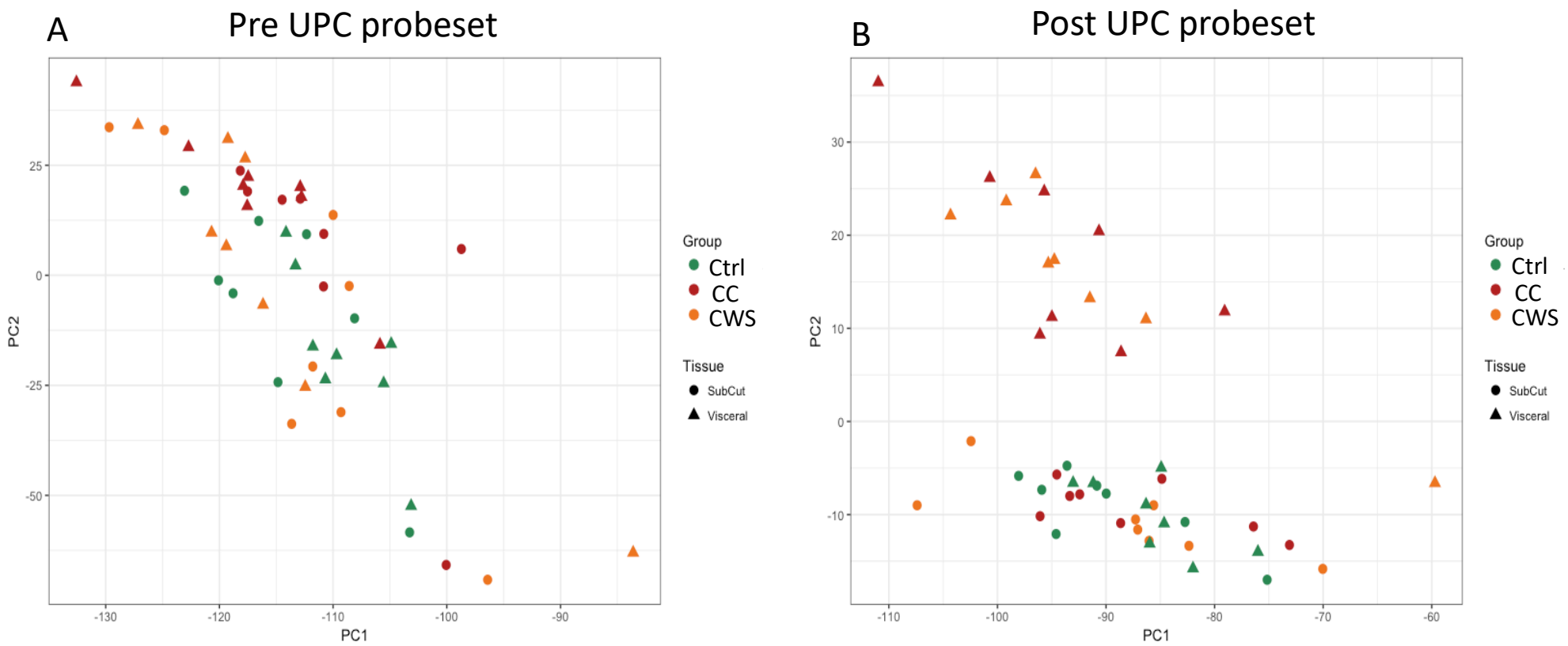


Figure 44 Adipose PCA analysis. Scatterplots showing separation of adipose depot and group samples by principle components analysis before (A) and after (B) removal of non/low expressed genes using the Universal exPression Code (UPC) (Piccolo, 2013) generated filter. Ctrl – healthy control. CWS – cancer weight stable. CC – cancer

In the CC group 2101 probesets were significantly regulated between the fat depots with fold changes ranging from 8-fold upregulated to 2-fold downregulated. In the CWS group 1722 probesets were significantly regulated with fold changes ranging from 9-fold upregulated to 2.5-fold downregulated. In the control group 1659 probesets were differentially regulated with fold changes from 4.6-fold upregulated to 2.3-fold downregulated. These data indicate that SAT and VAT depots have substantial mRNA expression differences. In addition the number of differentially expressed probesets is higher in the cancer groups and highest in the CC group (table 24). Comparing VAT between the groups found 2200 (10-fold up to 2.3-fold down) and 1253 (9-fold up to 2.3-fold down) probesets regulated between CC, CWS and control respectively. No probesets met the statistical cut off for significance in VAT between the CC and CWS groups.

	No. Regulated genes (FDR < 5%)
VAT v SAT in Group Comparison	
CWL VAT v SAT	2101
CWS VAT v SAT	1722
Control VAT v SAT	1659
VAT Between Group Comparisons	
CWL v Control	2200
CWS v Control	1253
CWL v CWS	0[1]
SAT Between Group Comparisons	
CWL v Control	0[2]
CWS v Control	0[3]
CWL v CWS	0[4]

Table 24 Regulated genes between groups. Number of genes regulated in the microarray data with FDR < 5% in each within and between group comparison.

1 – lowest adjusted p-value = 0.7; 2 – lowest adjusted p-value = 0.1; 3 – lowest adjusted p-value = 1; 4 – lowest adjusted p-value = 0.5.

Intelectin-1 was the most upregulated gene with a 10-fold increase in CC versus control and a 9-fold increase in CWS versus control (Figure 45). Intelectin-2 was also significantly increased across these comparisons. SAT demonstrated no significant differences in any comparison. These data demonstrate that whilst the transcriptional profile of SAT is not sensitive to the presence of cancer or cancer cachexia in our cohort there are substantial differences in VAT mRNA expression in the presence of cancer.

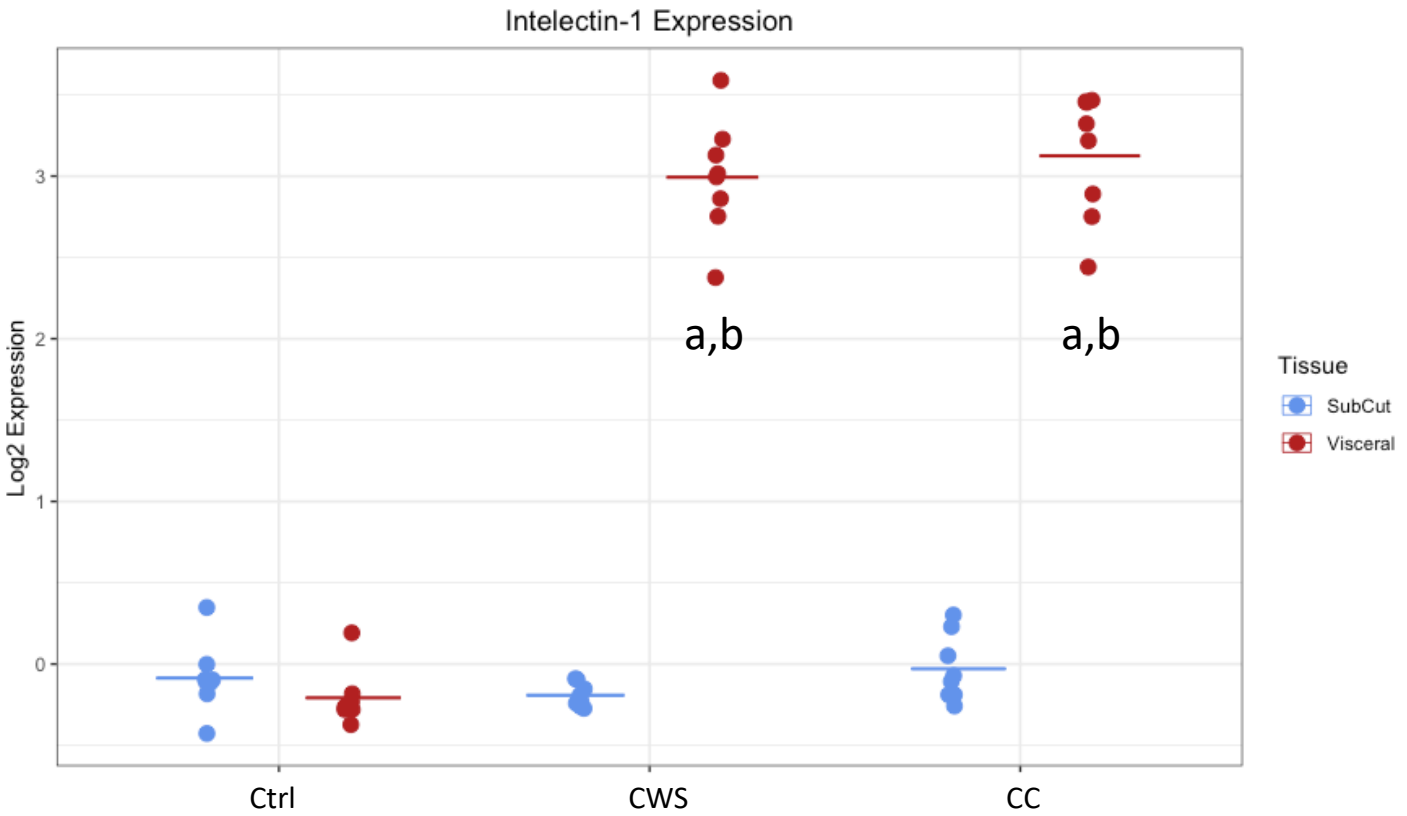


Figure 45 Intelectin-1 mRNA expression values (log2 transformed) from the microarray data. Ctrl = healthy controls, CWS = cancer weight stable, CC = cancer cachexia. a = significantly different from paired subcutaneous values; b = significantly different from control visceral values.

6.4.3 Geneset Enrichment Analysis

The Broad Institute Hallmark curated genesets collection (356) was used to examine the data for geneset enrichment. Firstly, differences between fat depots in each group were assessed. Seven genesets were significantly enriched in the CC group – Adipogenesis (FDR $2x10^{-9}$), Fatty acid metabolism (FD 0.004), Myogenesis (FDR 0.008), Hedgehog Signalling (0.044) and Oxidative Phosphorylation (0.047) were downregulated and Inflammatory response (FDR 0.036) and Allograft Rejection (FDR 0.043) were upregulated – in VAT compared to SAT. Two genesets were regulated in the CWS group – Adipogenesis (FDR $1x10^{-6}$) and Fatty acid metabolism (FDR 0.044) – both downregulated in VAT compared to SAT. There were 23 genesets regulated in the control group. Only Adipogenesis and Fatty Acid metabolism were enriched in all three VAT versus SAT comparisons. In cancer these genesets were downregulated whilst in the control group they were upregulated. The Inflammatory Response and Oxidative Phosphorylation genesets were common only between CC and control VAT versus SAT comparisons. The Inflammatory response category was increased in expression in both cancer cachexia and control VAT compared to SAT but different genes drove the enrichment signal in each group (Figure 46). The oxidative Phosphorylation genesets was downregulated in CC and upregulated in controls in VAT versus SAT. These data suggest a downregulation in genes related to growth of adipose tissue in cancer irrespective of weight loss whilst (compared to SAT) inflammation is increased in the VAT of cachectic patients.

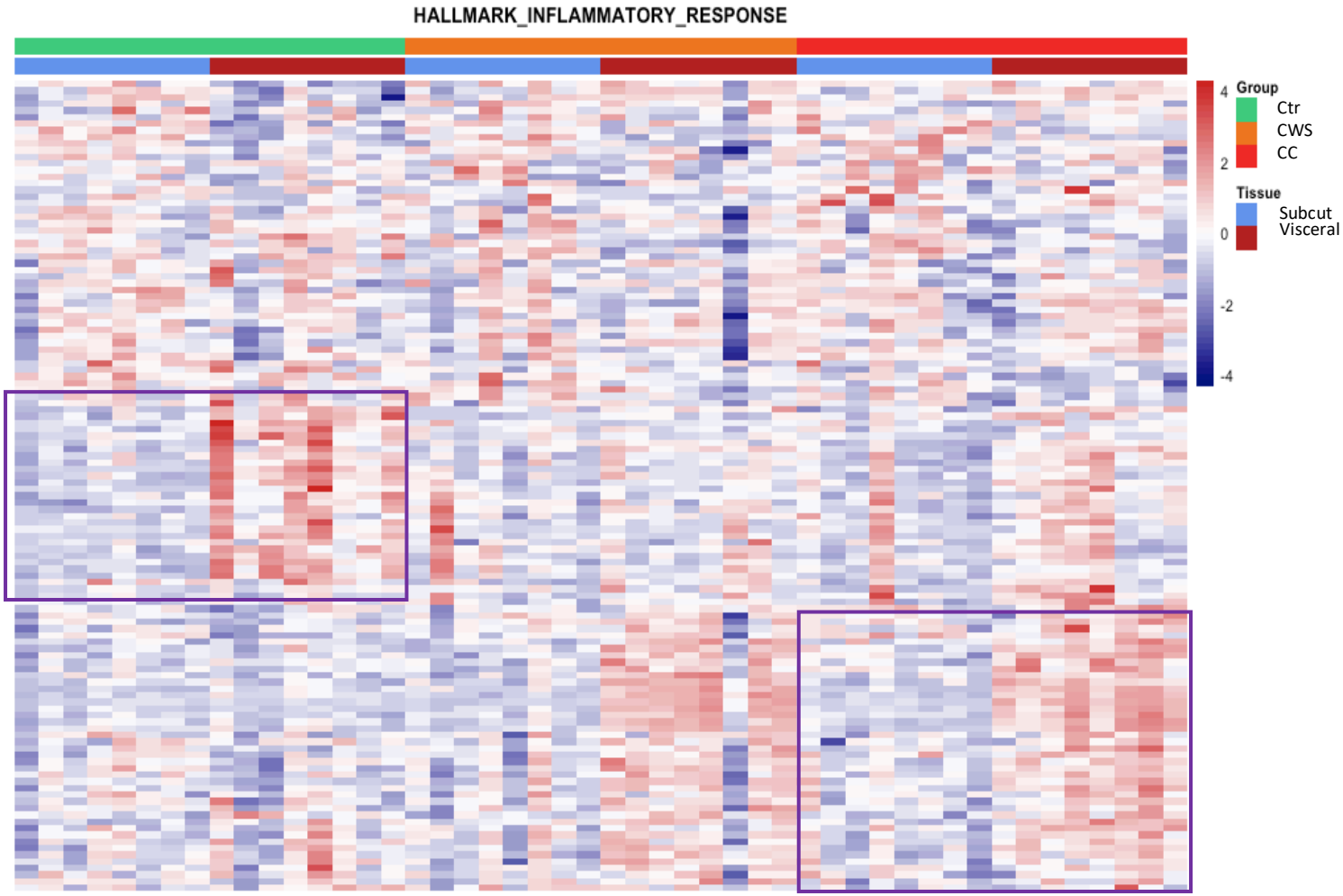


Figure 46 Heatmap of genes in Hallmark Inflammatory Response geneset. Data is subcutaneous and visceral adipose tissue of healthy control (Ctrl), cancer weight stable (CWS) and cachectic cancer (CC) patients. The purple boxes delineate inflammatory genes differentially contributing to geneset enrichment in cancer cachexia and controls. The cancer weight stable group demonstrate a similar signal to the cancer cachexia group but the geneset as a whole did not meet statistical significance for enrichment in VAT versus SAT.

Next, category enrichment in VAT between the groups was examined. In cachexia compared to control there were 14 categories enriched. Notably Oxidative phosphorylation (FDR $1.2x10^{-16}$), Adipogenesis (FDR $1.2x10^{-16}$) and Fatty acid metabolism ($3.3x10^{-9}$) were downregulated in CC compared to control. There were five categories enriched in the CWS group compared to control. MYC Targets (FDR 0.038) and Allograft rejection (FDR 0.046) were upregulated in the presence of cancer whilst Adipogenesis (FDR 0.038), Xenobiotic metabolism (FDR 0.046) and Hedgehog signalling (FDR 0.038) were downregulated in the presence of cancer. Whilst no individual mRNA met the statistical thresholds for significance in CC versus CWS VAT (see above) five genesets were significantly enriched – MYC targets (FDR 0.038) and Allograft rejection (FDR 0.046) were co-ordinately increased in CC whilst Adipogenesis (FDR 0.038), Hedgehog signalling (FDR 0.038) and Xenobiotic Metabolism (FDR 0.046) were co-ordinately downregulated. Given the observation of a downregulated energy metabolism signature in the VAT of cancer patients the Gene Ontology category Brown Fat Cell Differentiation (GO:0045444) was specifically examined and found to be downregulated in visceral adipose tissue in cachexia ($p = 5.4x10^{-6}$) and in weight stable cancer ($p = 2x10^{-7}$) compared to control. These data suggest that there is a downregulation of mRNA related to adipose expansion and energy use/generation in the VAT of cancer patients and that this is a stronger signature in CC patients. There was no evidence of brown fat cell differentiation in this cohort of CC patients.

QPCR was used to examine selected genes including those detected as regulated in the microarray data and the biological processes of fat browning, inflammation and adipogenesis.

6.4.4 Microarray candidates

ALOX15 demonstrated a significant group x depot interaction effect (p=0.005) with increased expression in VAT in the cancer groups compared to control (CC p=0.018 and CWS p=0.025 respectively). There was no difference between in ALOX15 expression in VAT between the cancer groups (p=0.9). ALOX15 was also increased in VAT compared to SAT in the cancer groups (weight stable p=0.04, cachectic p=0.0046) but not in the control group (p=0.9). ALOX15 was not differentially expressed in SAT depots between groups. These results are in agreement with the array results. There was a significant group x depot interaction effect for ITLN1 (p=0.0006). ITLN1 was significantly increased in the VAT of the cancer groups (CC p=0.0001, CWS p=0.02) compared to VAT in the control group but was not different between the cancer groups (p=0.5). ITLN1 expression was also significantly greater in VAT compared to SAT in the cancer groups (CC p=0.0004, CWS p=0.03) with no difference in the control group (p=0.9). There were no significant differences in ITLN1 in SAT between any groups. PPARA2 also demonstrated a group x depot interaction (p=0.004). However multiple testing was unable to detect specific differences. Inspection of the qPCR data suggests lower expression in the VAT of the cancer groups. The direction of change in the

qPCR data for the cancer groups was the same as seen in the microarray. The difference PPARA2 expression between the VAT and SAT depot in the control group was significant (p=0.03).

6.4.5 Genes involved in fat browning

There were no differences in UCP1 expression in terms of either an interaction (group x depot) or main effects. This is in agreement with the microarray results. PPARGC1A demonstrated a main effect of depot (p=0.003) with VAT showing higher expression than SAT. Post-hoc testing revealed that PPARGC1A was significantly upregulated in the VAT compared to SAT in the control group only (p=0.01). The direction of change for PPARGC1A in other comparisons was the same as seen in the microarray data but not statistically significant after multiple testing correction. There were no significant differences in PRDM16 expression in terms of interactions or main effects.

6.4.6 Inflammation

There were no significant differences in ACVR2A1 expression in terms of interactions or main effects but the direction of changes seen in the qPCR data agreed with the microarray data. There were no significant differences in IL6 expression in terms of interactions or main effects. IL6 was significantly downregulated in CC compared to control VAT in the microarray data. The direction of change was the same in the qPCR data driven by few patients (Figure 47) and not statistically significant. IL18 demonstrated a significant

group x depot interaction effect (p = 0.0009) with increased expression in the VAT of both cancer groups compared to control (CWS p=0.02, CC p=0.0001). Expression of IL18 was also increased in the VAT compared to the SAT in the cancer groups (CWS p=0.03, CC p=0.0006) but not in the control group (p=0.9).

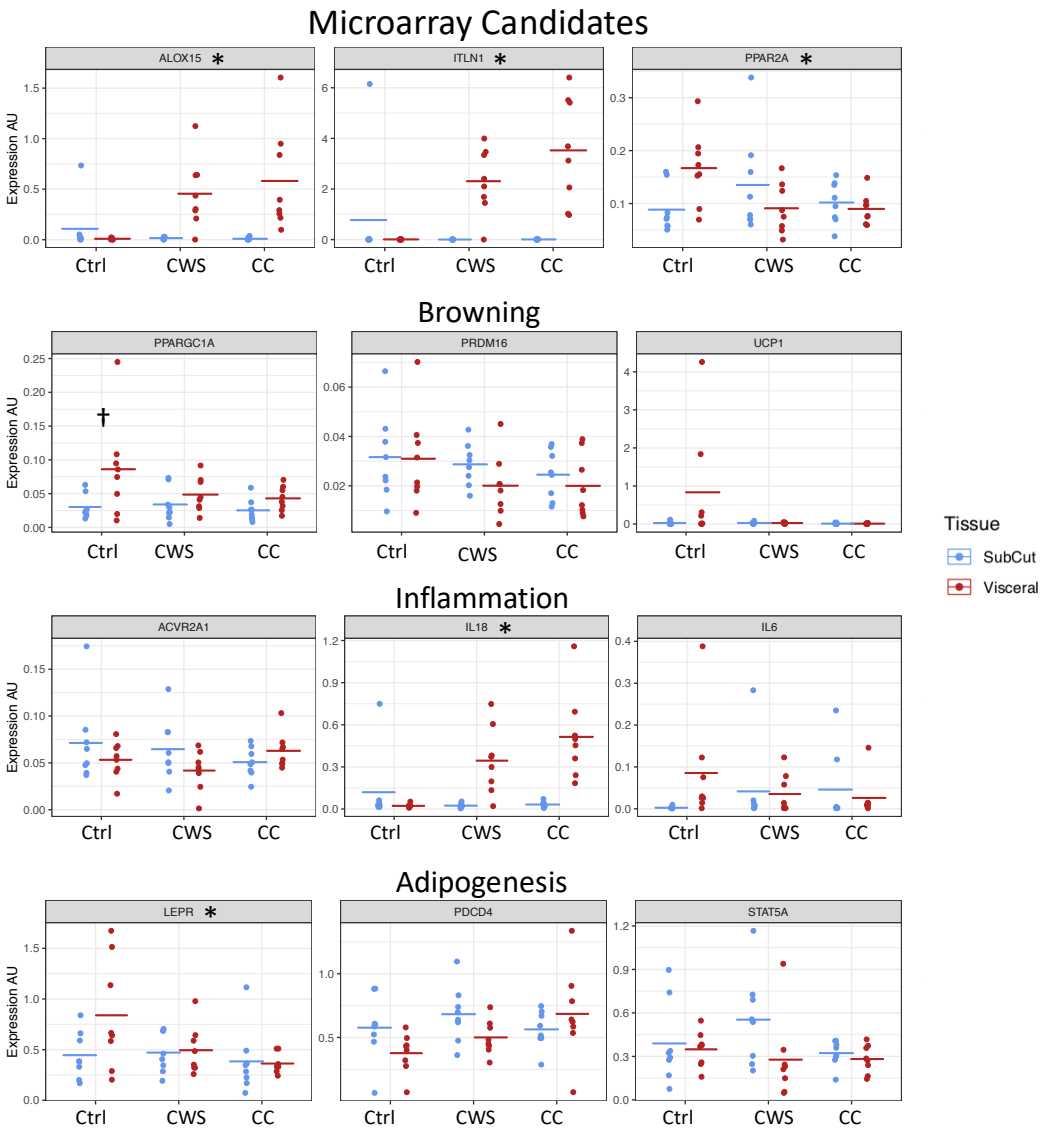


Figure 47 Data from qPCR of selected genes. Categories of genes validated by qPCR were - significantly different in microarray data (ALOX15, ITLN1, PPARA2), involved in browning (PPARGC1A, PRDM16, UCP1), inflammation related (ACVR2A1, IL6, IL18), involved in adipogenesis (LEPR, STAT5A, PDCD4). * = significant group x depot interaction; † = no significant interaction but significant difference between same depot in control group. See main text for full reporting of statistically significant differences. Ctrl = healthy controls, CWS = cancer weight stable, CC = cancer cachexia

6.4.7 Adipogenesis

LEPR demonstrated a significant group x depot interaction effect (p=0.03) with post-hoc testing revealing decreased expression in the VAT in the CC compared to control group (p=0.04). LEPR was also increased in control VAT compared to SAT (p=0.02). STAT5A did not demonstrate any significant interaction or main effects although the effect of fat depot was borderline significant (p=0.06) with specific differences undetectable by post-hoc testing. PDCD4 did not demonstrate any significant interaction or main effects.

6.4.8 ELISA results

VAT ITLN1 concentrations are shown in figure 48. Linear modelling of log ITLN1 concentration by group showed a significant effect of group (p=0.04). There was a significant difference between the CC and control groups (mean ITLN1 concentrations 43.6ng/ml and 20.5ng/ml respectively, p=0.014) but no statistically significant difference between CWS (mean ITLN1 concentration 30.9ng/ml) and either of the other two groups.

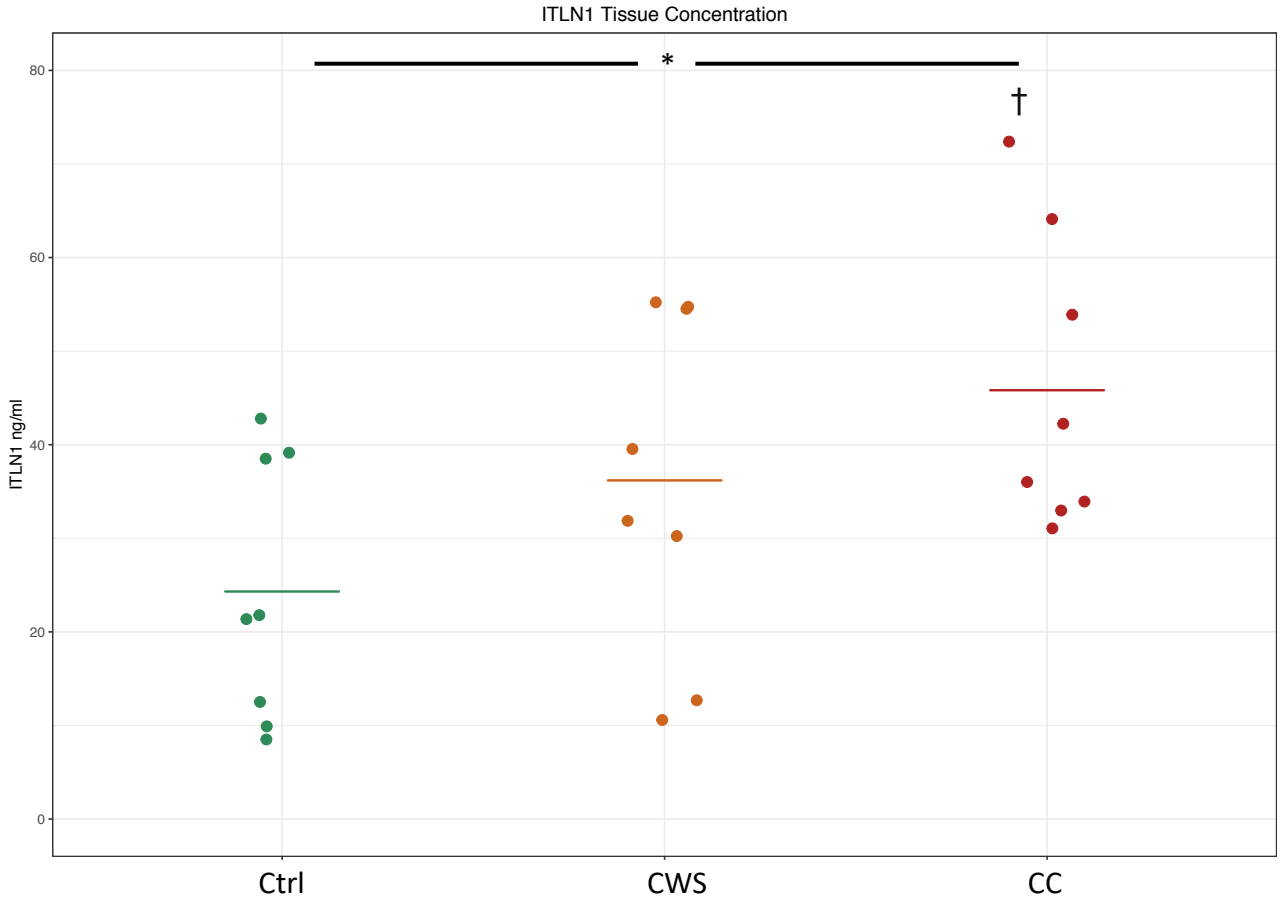


Figure 48 Intelectin-1 tissue ELISA. Intelectin-1 visceral adipose tissue protein concentration (ng/ml) as measured by ELISA across the control (Ctrl), cancer weight stable (CWS) and cancer cachexia (CC) groups. * = significant effect of group; † = significantly different from control group.

Patients demographics of those included in the plasma ELISA study are summarised in table 25.

Group (n=20)	Control	Cachexia	Weight stable	P value
Male:Female	14:6	14:6	14:6	/
Age (years)	49 (11)	69 (10)	64 (9)	<0.001
BMI (kg/m^2)	25.66 (2.81)	25.06 (4.85)	29.93 (7.79)	0.187
%weight loss	0	11.60 (6.58)	0.26 (0.71)	<0.001
SMI (cm^2/m^2)	50.69 (7.55)	41.54 (4.82)	55.92 (12.48)	<0.001
VATI (cm^2/m^2)	26.23 (20.04)	41.50 (36.65)	111.90 (215.25)	0.008
SATI (cm^2/m^2)	52.98 (27.79)	71.85 (59.75)	71.87 (43.75)	0.460

Table 25 Details of patients included in the plasma ITLN1 ELISA

Data are mean (SD).

Plasma ITLN1 concentrations are shown in figure 49. Mixed effects linear modelling with group as a fixed effect and assay run as a random effect was used to model log ITLN1 concentrations. There was a significant effect of group (p=0.028). Post-hoc testing using Tukey's correction demonstrated a significant difference in plasma ITLN1 concentrations between the cachectic and cancer weight stable groups (mean ITLN1 concentrations 2.6ng/ml and 1.9ng/ml respectively, p = 0.02). There was no significant difference between the control group (mean ITLN1 concentration 2.3ng/ml) and either of the other groups.

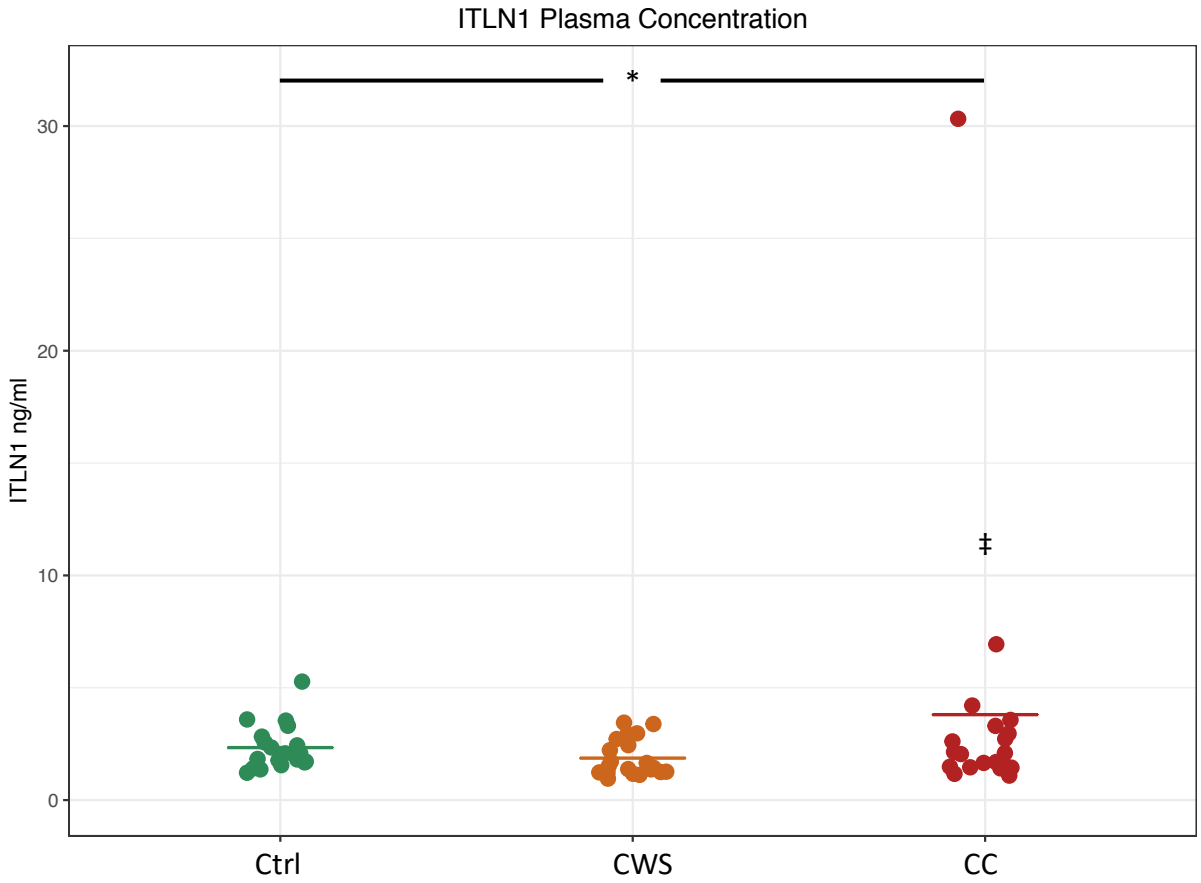


Figure 49 Plasma Intelectin-1 ELISA. Intelectin-1 plasma protein concentration (ng/ml) as measured by ELISA across the control (Ctrl), cancer weight stable (CWS) and cancer cachexia (CC) groups. * = significant effect of group; ‡ = significantly different from Ca group.

6.5 Discussion

Different mechanisms have been previously proposed to account for the changes in adipose tissue seen in cachexia. In CC, human and animal studies have shown increased rates of lipid mobilisation and loss of adipose tissue before any demonstrable changes in loss of muscle mass (360). An increased rate of adipose tissue loss has also been associated with worsening prognosis in cancer patients highlighting the importance of gaining a greater understanding of the mechanisms involved. To the best of our knowledge, this is the first study to examine both SAT and VAT depots in CC patients. This study has shown that SAT and VAT have unique gene expression signatures and VAT has an altered gene expression signature in cancer. It found increased expression of Intelectin-1 and Intelectin-2 in particular suggesting these adipokines may play a role in adipose changes in response to cancer. Intelectin-1 may be a target for therapeutic manipulation.

The gene which showed the largest difference in expression was ITLN1; a 34 kDa novel adipokine. Expression of ITLN2 was also high but not as great as ITLN1 and so ITLN1 formed the focus of this study. ITLN1 is preferentially produced by stromovascular cells in VAT(361) but has also been identified in human epicardial fat cells, mesothelial cells, airway goblet cells and cells lining the gut and ovaries (362). A series of studies have linked raised levels of ITLN1 to various cancers (265,363–369). ITLN1 has been suggested to promote cancer cell growth by triggering genomic instability via PI3K/AKt

(phosphatidylinositol-3 kinase downstream effector) signalling pathways (370). The role of ITLN1 in CC however, has not been well characterised. Randomised control trials have shown that weight loss significantly increases plasma ITLN1 concentration (371) whereas hyper-insulinaemic induction in healthy individuals reduces ITLN1 plasma concentration (372). Decreased levels of ITLN1 have been associated with obesity in patients with ovarian cancer (372) and levels of ITLN1 measured before patients were diagnosed with colorectal cancer have been confined to non-obese individuals suggesting it has a role in weight loss (373).

The microarray results confirmed that expression of ITLN1 was higher in VAT in cancer although not confined to CC patients. Plasma levels of Itln1 were significantly increased in CC compared to CWS groups but showed no difference with controls suggesting it possibly has a role in cancer induced weight loss. However as in the previous studies, this study found that plasma Itln1 differences were driven by a few individuals with high levels. The combination of low study numbers and high interindividual variability in plasma ITLN1 likely limited the power to detect an effect across all groups here. The significant increase in intelectin seen in the VAT ELISA of cachectic patients suggests a possible role in the pathogenesis of cachexia. There was a clear increasing gradient from controls through CWS to CC in VAT Itln1 level. This was not reflected in the plasma Itln1 protein levels. These results are similar to those previously seen with zinc a-glycoprotein (ZAG) (339) in CC. This

pattern of increased tissue protein levels but no change in circulating levels could be explained by cells outside adipose tissue contributing to circulating protein. Alternatively there could be a predominantly paracrine role for adipose tissue secreted protein which is not released into the circulation.

There was no evidence of increased browning in white adipose tissue in this cohort. Previously increased thermogenic activity of brown and beige adipose tissue has been shown to contribute to the increased energy expenditure and weight loss in rodent models of cachexia (374–376). White adipose tissue browning is associated with increased expression of uncoupling protein 1 (UCP1), which uncouples mitochondrial respiration toward thermogenesis instead of ATP synthesis, leading to increased lipid mobilization and energy expenditure (377). Whilst browning of white fat depots contributes to futile energy cycling in cachexia in animal models the importance of this is uncertain (378,379). Genes involved in fat browning in the present study were not upregulated suggesting browning has a limited role in the pathogenesis of cachexia in patients and highlights the importance of patient based research.

Cachexia is thought to be a chronic inflammatory state and changes in inflammatory mediators in adipocytes may be capable of inducing changes in adipose tissue homeostasis (380). Several studies have shown adipose tissue to be the target of several pro-cachectic factors as well as adipose tissue itself being an important source of inflammatory mediators (381–385). The majority

of studies assessing inflammation in cachexia have examined inflammatory markers from a systemic point of view rather than a tissue specific one with IL6 in particular frequently found to be raised in the plasma of CC patients (134). IL6 was downregulated in adipose tissue in CC suggesting a possible homeostatic response. Specific adipose tissue inflammation is important to consider as it may be an event very early on in the cachectic process prior to any systemic effects being demonstrated.

Adipogenesis is the process by which preadipocytes differentiate into mature adipocytes able to store triglycerides and secrete adipokines. Some studies have shown that adipogenic genes are downregulated in animal epididymal and retroperitoneal fat in cachexia (383,386). Co-culture studies have demonstrated inhibition of pre-adipocyte maturation in the presence of tumour cells which was associated with increased inflammatory cytokines (387). Changes in adipogenesis are likely to precede the clinical signs of fat wasting and tissue inflammation. It is possible therefore that factors such as increased infiltration of macrophages and production of inflammatory cytokines silence adipogenic genes and/or increase lipolysis leading to adipose wasting in cachexia.

6.5.1 Limitations

The current study confers similar limitations to other microarray studies most notably in the small sample size. However exploratory work of this nature is

valuable as it provides testable hypotheses to be taken forward. Another limitation of this study was the availability of omental and perinephric adipose tissue in the cancer and control, groups respectively. Differences in these two depots are not well defined. In rodents there are possible depot specific differences in innervation but this has not been documented in humans (388). A very small study in humans has suggested blood flow in omental fat may be higher than in perinephric fat though the difference was not significant (389). This may potentially the affect the removal of lipolytic products and the rates of lipolysis. The patients studied were predominantly male and we are therefore unable to draw conclusions about sex specific differences. There was no longitudinal follow up of patients and so it is unclear whether the patients in the CWS group went on to develop cachexia and therefore may have been pre-cachectic at the time of biopsy. We also excluded patients in the control and CWS groups who were sarcopenic on CT. In doing so we have assumed that a reduction in SMI in the cachectic group was due to disease. Given that these patients have a median age of 70 it is possible that they were sarcopenic prior to the start of the disease process.

6.6 Conclusion

This exploratory analysis confirms differential gene expression in adipose depots in patients with cancer with and without cachexia and highlights the importance of VAT in cancer. VAT may have a fundamental role in cachexia, but the downregulation of energy metabolism genes implies a limited role for

browning in cachectic patients as suggested in pre-clinical models. Future mechanistic studies are important to evaluate the effects of VAT in cachexia and in particular the role of Intelectin-1.

CHAPTER 7

PLASMA METABOLOMICS IN UPPER GASTROINTESTINAL CANCER

7.1 Chapter overview

The previous chapters have investigated methods of screening for cachexia along with providing an insight into potential pathophysiological methods of muscle and adipose wasting. The final chapter aims to tie these together and identify easily measurables markers of tissue wasting in the plasma of cachectic patients. Diagnosis as discussed is difficult and relies upon predominantly objective measures including weight loss, which often lags behind disease progression. The ability to diagnose cachexia early would lead to new avenues for treatment and improvement in prognosis. The 'omics' technologies allow us to study the end points of many biological processes. Among these, blood-based metabolomics is a promising method for cachexia research.

7.1.1 Objectives

This chapter aimed to perform liquid chromatography mass spectrometry (LC/MS) based metabolomics to investigate the metabolic profile of cancer cachexia.

7.1.2 Methods

Eighteen patients undergoing potentially curative surgery for UGI cancer were recruited. Fasting plasma samples were taken at the time of induction of anaesthesia. Samples were analysed blindly. Liquid chromatographic mass

spectrometry (LC/MS) analysis was undertaken. Data extraction for each of the samples was carried out by MZMine software. Metabolites obtained were evaluated manually and the quality of the peaks and retention times matched with standard metabolite mixtures. Univariate and multivariate analyses were performed using SIMCA-P 14.1 software.

7.1.3 Results

LC/MS analysis showed two distinct profiles, which clearly separated the patients into two groups (n=9 and 8 in each group) dependent on weight loss (greater or less than 5%). Overall 40 metabolites were associated with cachexia, 6 of those being highly discriminative of weight loss namely Lyso PC 18.2, L-Proline, Hexadecanoic acid, Octadecanoic acid, Phenylalanine and LysoPC 16:1.

7.1.4 Conclusion

This study gives biological validation to the consensus definition and provides a feasible diagnostic tool for identifying at-risk cachectic populations.

7.2 Introduction

As cachexia can develop progressively through various stages, from pre-cachexia to cachexia to refractory cachexia, early screening and staging is particularly important to prevent or delay its onset (28). An improved approach for detecting the evolution of muscle and or fat wasting would help target early intervention and treatment.

Recent progress in high-throughput analytical technologies and bioinformatics now permits simultaneous analysis of hundreds of compounds constituting the metabolome (390). Metabolomic analyses give complex fingerprints that appear to be characteristic of a given metabolic phenotype or diet. There have been very few previous studies attempting to quantify metabolites associated with cachexia. Those that have attempted to do so identified metabolites that are possibly discriminative of cachexia indicating there maybe scope for metabolomics based studies to identify biomarkers of cachexia (391–395).

Building upon the theory that metabolites produced from tissue breakdown are likely to be found in plasma and could potentially be a sensitive indicator of tissue wasting this study investigated whether metabolites associated with cachexia could be detected from the plasma of cancer patients. Urine proteomics have been previously used as a measure of degradation products in the circulating fraction allowing discrimination between weight losing and weight stable cancer patients (396). Plasma was selected as the biofluid of

choice for this study, since as previously shown several end products of muscle catabolism (e.g. creatinine and methylhistidine) can be easily measured here (397).

7.3 Methods

7.3.1 Participants

Patients with UGI cancer were recruited as previously described. CT body composition analysis was performed and fasting plasma samples were obtained at induction of anaesthesia approximately four to six weeks after the cessation of any neoadjuvant chemotherapy. Samples were stored locally at -80°C until transported to the metabolomic facility (Strathclyde Institute of Pharmacy and Biomedical sciences) in cool bags at -30°C.

7.3.2 Chemicals and Solvents

HPLC grade Acetonitrile (ACN) was purchased from Fisher Scientific (Loughborough, UK) and HPLC grade water was produced by a Direct-Q3 UltrapureWater System (Millipore, Watford, UK). AnalaR-grade formic acid (98%) was obtained from BDH-Merck (Poole, UK). Authentic stock standard metabolites (Sigma-Aldrich, Poole, U.K.) were prepared as previously described and diluted four times with ACN before LC-MS analysis. Ammonium acetate was purchased from Sigma-Aldrich (Poole, UK).

LC/MS Metabolomic analysis was performed by Dr Dave Watson and team at the University of Strathclyde

7.3.3 Sample preparation

Exactly 200 μL of the sample was mixed with 800 μL ACN containing 10 μg/ml of 2 ^{13}C glycine (Sigma-Aldrich, Poole, U.K.) as an internal standard to ensure retention time stability, and then centrifuged for 10 min before transferring into a vial with an insert. The pooled sample was prepared by pipetting 50 μL from each of the 18 samples and then mixing them together before diluting 0.2 ml of the pooled sample with 0.8 ml ACN containing 5 μg/mL of 2 ^{13}C glycine internal standard and centrifuging. Additionally, the prepared mixtures of authentic standard metabolites containing 10 μg/mL of 2 ^{13}C glycine as internal standard were run.

7.3.4 LC-MS conditions

Liquid chromatographic separation was carried out on an Accela HPLC system interfaced to an Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) using both a ZIC-pHILIC column (150 × 4.6 mm, 5 μm, HiChrom, Reading UK). The column was eluted with a mobile phase consisting of 20 mM ammonium carbonate in HPLC-grade water (solvent A) and ACN (solvent B), at a flow rate of 0.3 mL/min. The elution gradient was an A:B ratio of 20:80 at 0 min, 80:20 at 30 min, 92:8 at 35 min and finally 20:80 at 45 min. The nitrogen sheath and auxiliary gas flow rates were maintained at 50 and 17 arbitrary units. The electrospray ionisation (ESI) interface was operated in both positive and negative modes. The spray voltage was 4.5 kV for positive mode and 4.0 kV for negative mode, while the ion transfer capillary

temperature was 275°C. Full scan data were obtained in the mass-to-charge ratio (m/z) range of 75 to 1200 for both ionisation modes on the LC-MS system fully calibrated according to manufacturer's guidelines. The resulting data were acquired using the XCalibur 2.1.0 software package (Thermo Fisher Scientific, Bremen, Germany).

7.3.5 Data extraction and analysis

Data extraction for each of the samples was carried out by MZMatch software. The extracted ions, with their corresponding m/z values and retention times, were pasted into an Excel macro of the most common metabolites prepared in–house to facilitate identification. The lists of the metabolites obtained from these searches were then carefully evaluated manually by considering the quality of their peaks and their retention time match with the standard metabolite mixtures run in the same sequence. All metabolites were within 3 ppm of their exact masses. The list of metabolites was refined by removing all metabolites with RSD >20% in the pooled samples leaving a list of 318 metabolites. Statistical analyses were performed using both univariate with Microsoft Excel and multivariate approaches using SIMCA-P software version 14.1 (Umetrics, Umea, Sweden). The 318 metabolites were modelled to give a PCA plot and then supervised analysis based on OPLS was carried out refining the list of metabolites by eliminating the less important variables to give a model with the lowest possible number of variables. These data will be shared in an online database to increase the power of future studies.

8.4 Results

Plasma samples were analysed from UGI cancer patients (n = 18) taken from a cross-sectional cohort of UGI cancer patients who were recruited to two previously-published studies of muscle transcriptomics (n = 65 pre-surgical rectus abdominis biopsies, and n = 12 pre- and post-surgical resection muscle biopsies) (345,346). In these previous studies, quantitative significance analysis of microarrays produced an 83-gene signature that was able to identify patients with >5% weight loss, while this molecular profile was unrelated to markers of systemic inflammation. Comparison with healthy control muscle revealed that despite differences in the muscle transcriptome at baseline (941 genes regulated), the muscle of patients at post-surgical resection follow-up was similar to control muscle (two genes regulated). Baseline, pre-surgical plasma samples were available for 18 of these patients. Therefore, for the present study, there were 9 nine patients who had experienced ≥5% weight loss in the previous 6 months (≥5%WL group), and 9 nine patients with <5% weight loss (weight stable group: WS). Cancer cachexia had been primarily defined as ≥5% weight loss in order to identify patients who would have experienced dynamic wasting and thus be more likely to have identifiable markers and metabolic signatures. If the patients were analysed as two separate groups according to these weight loss criteria, the mean percentage weight loss in the ≥5% WL group was 14.39% compared with 2.13% in the WS group (p = 0.001). There were more males in the WS

group. There was no significant difference in age, BMI, SMI, SATI or VATI between the groups. However, patients in the ≥5% WL group did demonstrate higher CRP levels compared with WS patients. These body composition and systemic inflammatory phenomena are all associated with worsened outcomes in cancer patients (193,315,398), confirming the ≥5% WL patients as a high-risk group. Two patients in the WS group had >2% weight loss and low SMI [according to Martin criteria (27) on the CT]. Patients had a mixture of UGI cancers predominantly those of the oesophagus and pancreas (Table 26). On the overall metabolomic analysis, a total of 40 metabolites were significantly associated with ≥5% weight loss according to univariate analysis using a T test. The metabolites with the highest fold change were L-phenylalanine and various species of LysoPE, LysoPA and LysoPC. These metabolites, as well as free fatty acid levels (FFAs), were all elevated in the ≥5%WL group.

	Group 1 <5% weight loss (n=9)	Group 2 ≥5% weight loss (n=8)	P Values
Male: Female	8:1	4:4	N/A
Age (years)	61 (4.65)	66 (10.53)	0.167
% weight loss	2.13 (1.35)	14.39 (6.56)	0.001*
SMI (cm^2/m^2)	41.93 (16.78)	45.82 (7.72)	0.536
SATI (cm^2/m^2)	46.25 (20.38)	58.43 (33.86)	0.379
VATI (cm^2/m^2)	57.57 (55.28)	42.10 (33.48)	0.506
BMI (kg^2/m^2)	24.93 (4.42)	26.29 (4.64)	0.534
CRP (mg/l)	17.88 (27.06)	32.56 (50.44)	0.453
Cancer type	Pancreatic – 1 Oesophageal – 6 Gastric - 2	Pancreatic – 6 Oesophageal – 2 Duodenal - 1	N/A

Table 26 Metabolomic Patient details

All data are mean (standard deviation)
*Denotes significant result

Figure 50 shows the principal components analysis (PCA-X) of all 18 samples and 3 quality control (QC) samples which indicate the instrument stability was good for the duration of the run. PCA-X, an unsupervised model in SIMCA-P, produces a natural scatter of the samples based on their characteristic metabolomics footprints. In general, there was no separation of samples

according to the threshold defined above for cachexia as being > 5% weight loss.

Supervised models enable identification of metabolites that have the most significant contribution to a given clustering pattern. In SIMCA, supervised analysis can be carried out using orthogonal partial least squares discriminant analysis (OPLS-DA) or orthogonal partial least squares (OPLS) models. An OPLSDA model was not very successful in classifying the samples without omitting several. AN OPLS model based on six metabolites (Table 27) was successfully produced for all but one of the one of the cachectic samples. This sample was omitted from the OPLS model since it clustered with the weight stable samples. The model plotted predicted against actual weight loss and had a CVANOVA of 0.0014. The correlation line had an R^2 value of 0.976 when fitted through the samples (figure 51).

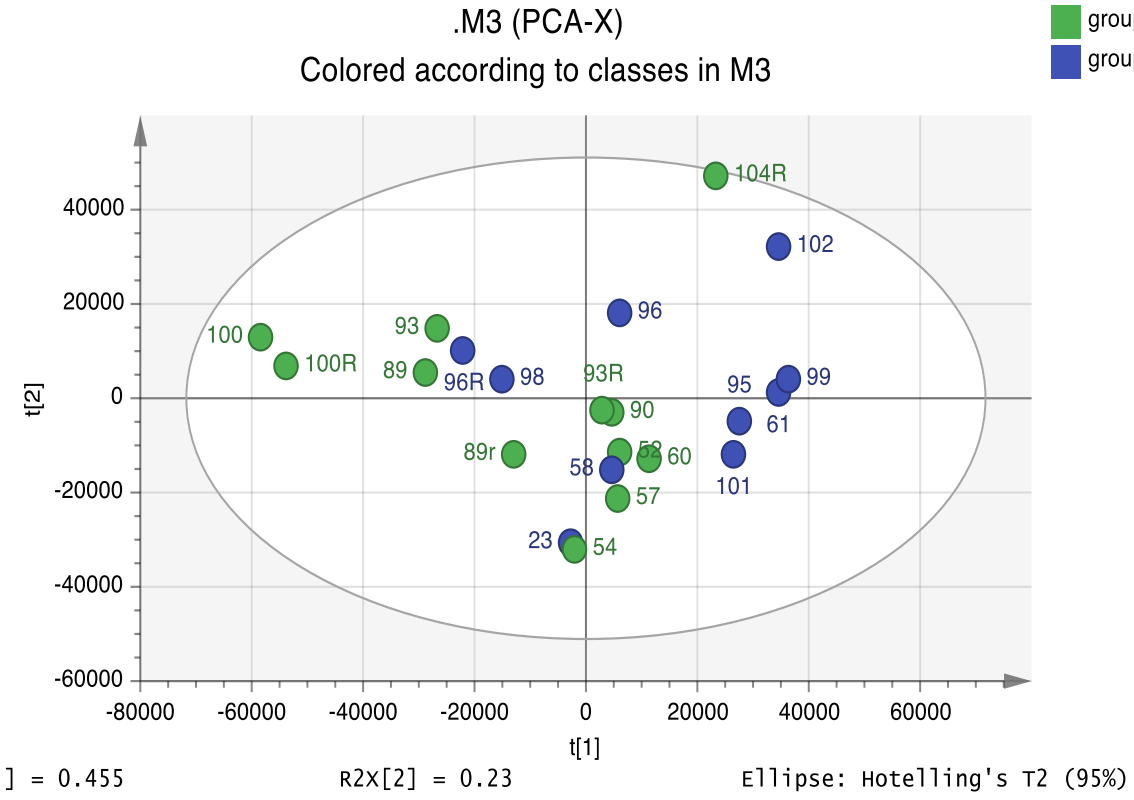


Figure 50 PCA-X analysis of the metabolomics footprint.18 plasma samples with pooled samples removed.

Green circles (group 1) = weight stable, blue circles (group 2) = weight losing

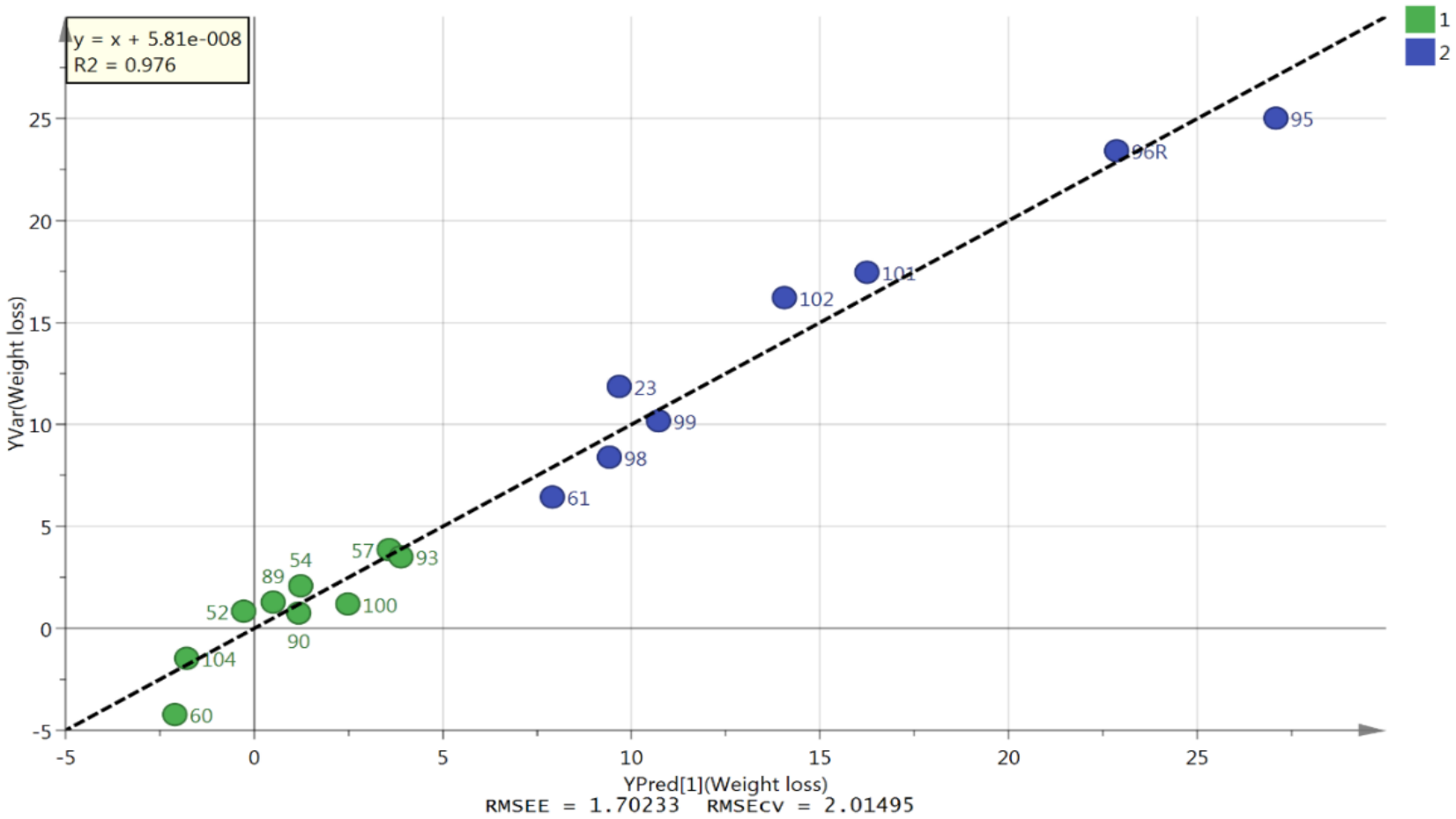


Figure 51 Orthogonal partial least squares (OPLS) model. Model showing close correlation for 8 weight losing samples and 9 weight stable samples between predicted and actual weight change

(CVANOVA = 0.0014) based on six variables listed in table 27

m/z	Rt (min)	Metabolite	VIP value
520.339	4.4	Lyso PC 18.2	1.82
116.071	13.0	L-Proline	1.43
255.233	4.3	Hexadecanoic acid	0.54
281.249	3.8	Octadecenoic acid	0.42
166.086	10.0	Phenylalanine	0.36
480.344	4.4	LysoPC 16:1	0.20

Table 27 The six metabolites used to produce the OPLS model (as shown in figure 51)

m/z- mass to charge ratio, rt – retention time

Table 28 shows the metabolites that were found to be significantly different between the two weight group categories. The ratio represents the intensity of the metabolites relative to the “weight stable” patient group.

Polarity	m/z	Rt(min)	Metabolite	P val	Ratio WL/WS
Amino acids					
P	116.071	13.0	L-Proline	0.015	1.36
P	166.086	10.0	L-Phenylalanine	0.619	0.87
Fatty acids					
N	214.048	4.3	sn-glycero-3-Phosphoethanolamine	0.006	1.78
N	255.233	4.3	Hexadecanoic acid	0.049	1.21
N	277.217	3.9	Octadecatrienoic acid	0.010	1.60
N	279.233	4.2	Linoleate	0.002	1.36
N	281.249	3.8	Octadecenoic acid	0.023	1.22
N	293.249	4.2	Nonadecadienoic acid	0.019	1.24
N	303.233	4.1	Eicosatetraenoic acid	0.022	1.37
N	305.249	4.2	Eicosatrienoic acid	0.054	1.50
N	327.233	4.2	Docosahexaenoic acid	0.025	0.81
N	329.249	4.1	Docosapentaenoic acid	0.009	1.46
N	331.264	3.9	Docosatetraenoic acid	0.014	1.68
P/N	380.255	5.1	Sphingenine phosphate	0.033	1.28
Lipids					
N	214.048	4.3	Glycerophosphoethanolamine	0.006	1.78
N	381.205	4.6	LPA 14:0	0.010	1.73
N	393.241	4.4	LPA16:0 ether	0.048	1.37
N	433.236	4.4	LPA 18:2	0.001	1.67
N	435.252	4.5	LPA18:1	0.006	1.40
N	437.267	4.2	LPA 18:0	0.028	1.23
P/N	454.292	4.6	LPE 16:0	0.040	1.44
N	457.235	4.3	LPA 20:4	0.001	1.62
N	464.278	4.4	LPC 14:1	0.007	1.36
P	468.308	4.6	LPC 14:0	0.026	1.58
P/N	476.278	4.4	LPE 18:2	0.013	1.98
P/N	478.292	4.4	LPE 18:1	0.013	2.02
P/N	480.308	4.4	LPE 18:0	0.012	2.07
N	485.267	4.3	LPA 22:4	0.007	1.96
P/N	496.339	4.4	LPC 16:0	0.040	1.34
N	498.262	4.3	LPE 20:5	0.053	2.13
P/N	500.278	4.4	LPE 20:4	0.002	2.36
N	504.31	4.4	LPE 20:2	0.002	1.65

N	514.294	4.3	LPC 18:4	0.005	2.28
P/N	520.339	4.4	LPC 18:2	0.001	1.75
P/N	524.278	4.3	LPE 22:6	0.032	1.45
N	526.294	4.3	LPE 22:5	0.004	2.51
N	528.31	4.3	LPE22:4	0.004	2.01
P/N	544.338	4.3	LPC 20:4	0.014	1.81
P/N	546.354	4.3	LPC 20:3	0.026	1.91
P	570.356	4.2	LPC 22:5	0.009	1.89
P	731.605	4.2	SMd18:0/18:1	0.052	1.29

Table 28 Significant metabolites that differ between weight loss vs. weight stable groups (comparing 9 weight stable and 8 weight losing patients)

PE - phosphatidyl ethanolamine, PC - phosphatidyl choline, PA - phosphatidic acid, MZ – mass to charge ratio, rt – retention time, P – detection in positive ion mode, N – detection in negative ion mode

Figure 52 shows a heat map showing the relative abundance of the lyso lipids in these plasma samples. Lyso PC18:2 is almost as abundant as lyso PC 16:0 and is elevated by 1.75 fold. Beyond these two lysolipids the response is much lower but there are many more minor lipids showing similar or greater fold changes in the weight losing patients.

m/z	Rt min			Group 1	Group 2
496.339	4.4	C24H50NO7P	LysoPC 16:0		
520.339	4.4	C26H50NO7P	LysoPC18:2		
544.338	4.3	C28H50NO7P	LysoPC20:4		
435.252	4.5	C21H41O7P	LysoPA18:1		
464.278	4.4	C22H44NO7P	LysoPC 14:0		
546.354	4.3	C28H52NO7P	LysoPC20:3		
437.267	4.2	C21H43O7P	LysoGP 18:0		
480.344	4.4	C24H50NO6P	Lyso PC 16:1		
478.292	4.4	C23H44NO7P	LysoPE18:2		
570.356	4.2	C30H52NO7P	LysoPC22:5		
480.308	4.4	C23H46NO7P	LysoPE18:0		
454.292	4.6	C21H44NO7P	LysoPE 16:0		
526.292	4.3	C27H44NO7P	LysoPE22:5		
457.235	4.3	C23H39O7P	LysoPG 20:4		
524.278	4.3	C27H44NO7P	LysoPC18:0		
508.341	4.3	C25H52NO7P	LysoPE 18:0		
504.31	4.4	C25H48NO7P	LysoPE20:2		
485.267	4.3	C25H43O7P	LysoPA20:4		
363.158	4.4	C16H29O7P	LysoPA 16:0		
514.294	4.3	C26H46NO7P	LysoPC18:4		
528.31	4.3	C27H48NO7P	LysoPE22:4		
540.309	4.4	C28H48NO7P	Lyso PC 20:5		

Figure 52 Heat map showing relative levels of lysolipids

purple= > 30%, yellow >1% blue >0.1%

m/z – mass to charge ratio, rt – retention time

8.5 Discussion

In this study, LC-MS based metabolomics analysis was performed to reveal the metabolic profile of weight loss in cancer. It was able to demonstrate distinct profiles associated with the presence or absence of ≥5% WL. Most of the metabolites identified within these profiles fell within the lipid pathways. The clearest finding was that several long chain fatty acids and lysolipids were elevated in the plasma of the patients with higher weight loss. Lysolipids are very abundant in plasma (399) and Lyso-PC 16:0 was the most abundant compound by response in this set of samples; an increase of 1.34 fold between ≥5% WL and WS patients represents a major shift in metabolic output.

The OPLS model shown in Figure 51 indicates a strong association between weight loss and six metabolites. Two of the metabolite markers were lysolipids, two were amino acids, and two were fatty acids. There is a risk of overfitting data when the numbers of samples are small. However, in the present study, there was a clear indication that a small number of markers may be used to model the degree of weight loss with accuracy. One patient with ≥5%WL was removed from the analysis as this sample clustered with the WS group, but for this small cohort alone, six metabolites, were identified as a diagnostic test for cachexia (≥5%WL), making it 95% accurate. Validation of these markers will require larger studies, ideally with sequential assessment. The current study therefore demonstrates an association between lipolytic activity in the plasma

of cancer patients with weight loss. The changes in the amino acids found add credence to the importance of muscle wasting in cancer cachexia and indeed, most current research into cancer cachexia focuses on this area. However, the importance of lipid metabolism is re-emerging as an area of priority (335).

Previously, cachexia in patients with cancer of the oesophagus and pancreas has been linked with high levels of plasma glycerol and free fatty acids (400,401). Weight-losing cancer patients have been shown to have increased turnover of both glycerol and fatty acids compared with cancer patients without weight loss (402). Some, however, have suggested that observed increases in lipolysis and triglyceride-fatty acid cycling in cachectic patients with oesophageal cancer are due to alterations in nutritional status rather than disease presence (403). Cachectic ovarian cancer patients have been shown to have increased levels of free fatty acids, monoacylglycerides and diglycerides in their serum and ascitic fluid (404). Whilst it is difficult to determine where fatty acid and lipid metabolites originate, both lysolipids and fatty acids are markers of lipolysis (108,405). Free fatty acids may also provide energy for the tumour, with the glycerol molecules released during the breakdown of triacylglycerides being used for gluconeogenesis by the liver (406,407).

Previous attempts to profile metabolites associated with cachexia have yielded varying results and differences in important metabolites produced in each

study. Metabolomics research in cachexia began in 2008 in the C26 mouse model (391). This was the first study to demonstrate a distinct metabolomics-based profile associated with the onset of muscle wasting, and identified increased levels of very low and low density lipoprotein associated with aberrant glycosylation of β-Dystroglycan (β-DG, a marker of muscle wasting in this mouse model) (49). Human metabolomics studies in cancer cachexia have investigated urine and plasma from weight-losing patients. These studies also found large numbers of glycerophospholipids and metabolites associated with amino acid metabolism and were able to identify occult sarcopenia in patients with cancer (392,408). Recent studies have attempted to separate pre-cachectic, cachectic and weight stable cancer patients and healthy controls using serum metabolomics, and enabled identification of fifteen highly discriminative metabolites (394). The present study was able to discriminate weight-losing from weight- stable patients using only six metabolites. The only study to find a markedly different metabolic pattern to the present study analysed patients using three analytical platforms, namely gas chromatography mass spectrometry, capillary electrophoresis mass spectrometry, and LC/MS. The authors found a significant reduction in amino acids and glycerophospholipids associated with cachexia, a difference that has not previously been described in this condition, plus a high increase in cortisol levels (395). All bar one of these studies (392) used 5% weight loss as a cut off to stratify patients, in a similar fashion to the present study. Future studies should investigate the relationship between metabolomics and

dynamic assessments of tissue loss over time, e.g., using serial CT body composition analysis.

Blood-based metabolomics is a promising method for cachexia research. However, as seen previously, results are often difficult to replicate due to the heterogeneity of the populations and study sizes. All the patients included in the present study were suitable for potentially curable surgical resection of their cancer and therefore had localized/non-metastatic disease and similar measures of muscle volume, indicating that the identified metabolites might be early markers of fat wasting preceding muscle loss. This hypothesis is supported by the fact that two of the patients in the WS group were “cachectic” according to the consensus definition, by virtue of having ≥2%WL and low SMI on CT, and yet, they did not group with the identified panel of six metabolites. This finding may be explained by a lack of dynamic wasting, and that CT-identified sarcopenia may be the pre-cancer patient norm, rather than a consequence of disease.

One obvious limitation of this study is the small number of samples used, involving differences in sex ratios and cancer types between the groups. This was an exploratory study involving patients without refractory cachexia and was not designed to identify sex- or tumour-specific differences. This study was limited to patients with UGI cancers and therefore, may not be applicable to all cancer types. Previous serum metabolomic studies in pancreatic and oesophageal cancer have confirmed that dysregulation of lipid metabolism is

a key component of these conditions, although the exact tissue site of these processes is unknown (409,410). In the present study, differences in tumour type between patient groups are unlikely to explain the identified six-metabolite signature, due to the close correlation with patient weight loss. It was able to demonstrate distinct metabolic profiles consistent with the consensus cachexia definition of ≥5% weight loss. These findings support previous muscle transcriptomic studies, and they give further biological validation to the 2011 Fearon consensus definition of cancer cachexia (28) as a valid patient inclusion criterion in clinical trials. Furthermore, these results provide a 6-metabolite profile for further investigation as a marker of cachexia in longitudinal studies, with the opportunity to explore early diagnosis and response to therapeutic intervention.

8.6 Conclusion

These results show that metabolomic profiles in plasma from cancer patients are different between patients with ≥ or <5% weight loss. Most of the metabolites identified within these profiles fell within the lipid pathways. Differences highlighted in the breakdown of lipids provide an understanding of the mechanisms involved in the pathogenesis of cachexia. A 6-metabolite signature correlated strongly with degree of patient weight loss. A better understanding of the importance of adipose wasting and the potential sharing

of datasets between studies may identify novel biomarker strategies and therapeutic approaches for cancer cachexia.

CHAPTER 8
GENERAL DISCUSSION

8.0 General Discussion

8.1 Overview

Cancer cachexia significantly impairs QoL and leads to poor surgical and oncological outcomes. This thesis set out to investigate current difficulties in characterising cancer cachexia as well as understand some of the currently underexplored pathophysiological mechanisms which underly muscle and adipose wasting. Each chapter has discussed the main findings for each study. This discussion aims to summarise the findings and place them into context within the overall thesis (figure 53).

Chapter 3 began by investigating the ability to screen for conditions which present with unintentional weight loss; namely cachexia, sarcopenia and malnutrition. Here, the difficulties in identifying patients with cachexia first become apparent. A contemporary review of the literature was performed using search engines encompassing a wide range of medical and importantly nursing and allied health professional journals. Search terms were included to make this review as broad as possible and in doing so several thousand papers were initially identified. It became apparent that many tools had been developed to screen for malnutrition but very few existed to identify patients with cachexia or sarcopenia and indeed it was not possible to identify one tool which is currently able to achieve screening for all three conditions. With the existence of so many tools already in operation it seems unlikely that the development of a completely new tool will lead to improved assessment.

Therefore suggested steps were included which would allow the enhancement of existing tools to encompass the diagnostic criteria for cachexia. This study importantly highlights the difficulties in screening for cachexia in the outpatient setting therefore leading to difficulties in recruiting appropriate patients to clinical trials or allowing targeted treatment of the correct underlying condition which has presented with unintentional weight loss. This chapter set the scene for the ongoing investigations carried out in this thesis. By detailing how difficult it is to identify patients with cachexia more robust methods are needed to allow early targeting of patients.

Chapter 4 therefore built on the assessment of cachexia and looked in more depth at radiological methods for diagnosing low muscle mass; namely CT. Indeed one of the main themes running throughout this thesis is the use of CT to quantify muscularity and adipose tissue volume. CT is a useful assessment of muscle mass in cancer patients as it forms part of routine care. CT does however, have certain limitations including exposure to radiation (81milligray per centimetres for a CT chest, abdomen and pelvis) and accessibility of measurements of muscle mass to clinicians. Factors such as patient position and scan quality can impact body composition assessment but when used correctly have been shown to provide a reliable assessment of muscle and adipose volumes (27). It was therefore used in most chapters to provide a reliable assessment of muscle mass and to divide patients into study groups based on the consensus definition.

Muscle mass may be measured by many means and broadly has been defined as less than 2 SD below the mean of a healthy population. It is therefore not necessarily applicable to all and it does not necessarily correlate with muscle function. This chapter therefore set out to investigate the assessment of body composition by age and sex in patients with oesophageal cancer. As expected older patients had a lower staging SMI and all patients were found to lose muscle mass following chemotherapy but somewhat surprisingly this did not differ by age. Baseline adipose tissue volume also differed by age and sex and older patients and females lost significantly more adipose tissue following chemotherapy. The type of adipose tissue lost was different by sex with males losing more visceral fat. The relative importance of fat wasting in cachexia is not well understood but this chapter has highlighted differences in adipose depots which require more biological investigation.

The patients included in this study were chosen as they had been previously recruited to cachexia research within the region and had undergone muscle biopsy. These patients were therefore felt to be representative of the target population. Many studies investigating CT derived muscle wasting include patients of both sexes over a wide age range with a variety of cancer types. The ability of studies investigating CT derived body composition to generalise results to differing patient populations must therefore be questioned. As with all CT based studies the assessment of body composition is only valid at the

time point at which the scan was undertaken and therefore does not provide a dynamic assessment of wasting. The number of patients in each group was very different with there being lower numbers at extremes of age. This likely reflects real life clinical practice but could be investigated further with a larger cohort. The study was also not powered to assess clinical outcomes and further research should investigate the association of CT derived body composition volumes with patient focused factors such as QoL, functional status and responses to treatment.

Linking back to chapter 3 chapter 4 begs the question as to whether CT should become a routine part of the screening process for cancer patients? With the ease of accessibility and the advancement of body composition analysis techniques it could feasibly be built into routine practice in particular to identify those patients who may benefit from increased pre-operative prehabilitation. In order to do this stringent diagnostic criteria needs to be developed for all populations and perhaps needs to be disease as well as patient specific. Given that it is currently the most robust way to identify patients with low muscle mass it formed the basis of phenotyping patients for the following two chapters. The differences in age and sex seen here however, were not used as a basis for recruitment of the following patients.

Chapter 5 was the main investigation into muscle wasting in this thesis and in particular the possible role of the NMJ in cancer cachexia. Evidence relating

to this had previously been derived from animal models of sarcopenia which had suggested that the NMJ is unstable in this condition. This chapter provided the not only the first characterisation of the NMJ in patients with confirmed cancer cachexia, it also provided the first characterisation of the NMJ in human disease. Findings were very different to those in rodent models showing that the NMJ is stable in cancer cachexia and thus it is likely to be a primary muscle pathology. Small numbers of patients were included in this chapter, pertaining to the labour intense process of NMJ characterisation in this cohort. Rectus muscle biopsies often yielded no NMJs meaning that many patients had to be recruited to obtain the numbers seen (n=60). Despite this several hundred NMJs were analysed for each, therefore providing a robust assessment. Some of the weight stable patients did however have low muscularity on CT possibly due to ageing that often co-exists with cancer associated muscle loss and current diagnostic criteria is unable to separate one from the other. A previous study confirmed that when this definition of 2% weight loss and low muscularity was used muscle fibre size, protein content and RNA/DNA content were all reduced therefore confirming histological muscle wasting more so than other definitions available (312). Groups were also not age or sex matched meaning that as shown in the previous chapter baseline body composition could have been markedly different for this reason alone. This was not undertaken due to the difficulty in recruiting suitable patients and obtaining NMJ's in each sample. If the study was to be repeated on a larger scale this should be taken into account.

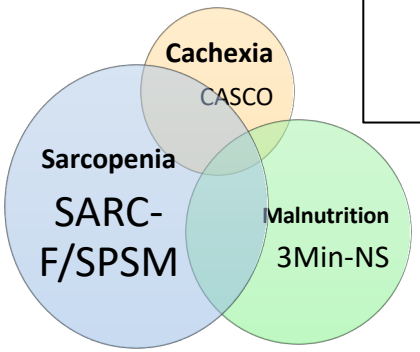
Chapter 6 returns to adipose tissue. Chapter 4 for the first time demonstrated that different adipose depots show different rates of wasting. This exploratory analysis therefore took tissue samples of these depots to investigate the first study of this nature in humans with cancer cachexia. It confirmed differential gene expression in adipose depots in patients with cancer with and without cachexia and highlighted the importance of visceral adipose tissue in particular. Intelectin-1 was identified as a novel adipokine in the visceral adipose of patients with cancer, having been shown to be previously linked to weight loss in healthy people. It was not successfully found to be raised in the plasma of cachectic patients limiting its role as a potential biomarker for the disease. Tissue ELISA levels were however raised indicating it may have an autocrine effect. There was also a down regulation of genes involved in energy metabolism in particular in fat browning suggesting it has a limited role in patients compared with animal models and again showing the need for future patient based studies. The main limitations of this chapter were in the difference in visceral adipose depots used (omental and perinephric fat for the cancer and control patients respectively). Omental fat was unable to be sampled from the control patients due to the nature of the operation. Future studies could look at sampling omentum from other control groups e.g. patients undergoing hernia repair to eliminate this possible reason for differences seen. The study should also be repeated in a much larger cohort of patients with high significance values in order to dampen down the signal from a large number of markers unlikely to be associated with the disease.

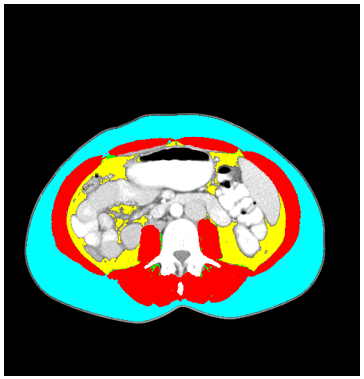
The final chapter of this thesis investigated the ability of metabolomics based analysis to identify markers of tissue wasting in the plasma of cancer patients. It was able to identify 6 metabolites that were highly discriminative of weight loss namely Lyso PC 18.2, L-Proline, Hexadecanoic acid, Octadecenoic acid, Phenylalanine and Lyso PC 16.1. Interestingly again this study produced more markers of adipose tissue wasting, in particular lipolysis-promoting activity. This was the only study in the thesis not to use CT based assessment of muscle wasting. The patients in this cohort were pre-selected as they had already been involved in a transcriptomic study making their categorisation into two groups based on CT difficult. The modelling that was performed demonstrated that patients were able to be identified using a different part of the consensus definition (>5% weight loss). It is possible that given the differences in rates of sarcopenia seen by age and sex in chapter 4 that these results may have been different depending on the way the patients were characterised from the outset. Interestingly fat breakdown products were seen in the plasma of patients in this study but itln1 was not seen in the plasma of patients in the previous study. This was due perhaps to large variability in individuals. The plasma was re-analysed in chapter 6 by weight loss categorisation of cachexia and did not appear to make any difference suggesting the definition of cachexia used had no impact on the results. This study also contained small numbers of patients but showed that there is the potential to use these numbers to classify patients based on their degree of cachexia. Validation of the metabolites seen would be required in a larger cohort.

Figure 53 Summary of thesis

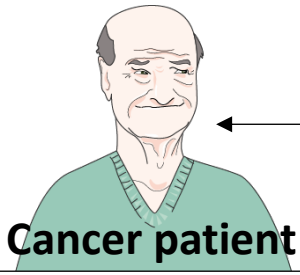
Characterisation of cachexia

- Current screening methods inadequate
- Unable to differentiate between conditions that cause UWL
- Better tools required to identify patients suitable for entry into trials

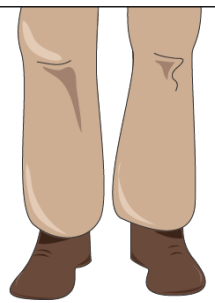




- CT currently gold standard for analysing body composition
- Muscle and adipose wasting varies by age and sex
- Should it become part of routine cancer care?

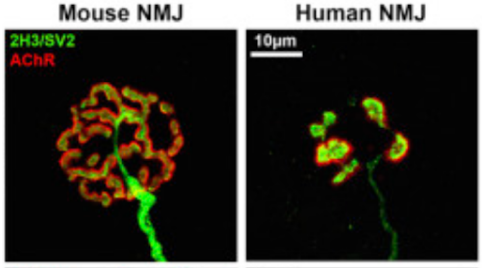


-Increased ability to identify those suitable to enter trials
-Screening of patients involving CT needs to be patient and disease specific
-Novel therapeutic strategies such as neural stimulation may be of use in the future
-Possible new mediators and biomarkers of adipose wasting could monitor response to treatment.



Mechanisms of cachexia

- NMJ's are stable in cancer cachexia
- No evidence of denervation
- Muscle remains primary locus of pathology in cachexia



- Large differences in gene expression between VAT and SAT
- VAT metabolically active in cancer
- Intelectin-1 possible mediator of cachexia
- Human adipose tissue behaves differently to rodents

- Patients able to be separated based on % weight loss
- Lipid breakdown products identified in plasma of cachectic patients

8.2 Future thoughts

The majority of the studies included in this thesis included small patient numbers. In order to fully validate the findings they would need to be repeated on a larger scale and in patients with other types of cancer to ensure that the results found are not exclusive to those with UGI cancers. Whilst this thesis has produced some interesting results and has contributed to the body of cachexia knowledge, several questions remain unanswered and require further thought.

This thesis has demonstrated clinical adipose wasting by CT. It has also analysed the mechanism for adipose wasting by transcriptomics and potentially identified downstream degradation products of this adipose wasting process by metabolomics. This linkage suggests that breakdown products of fat wasting do end up in the circulating compartments. Does this indicate that adipose cell membranes become disrupted during lipolysis and cachexia as they do in muscle cells (411)? Is it possible that the same degradation products could be found in urine thus leading to the discovery of potential non-invasive urinary biomarkers? The link between fat and muscle wasting may also be investigated by looking at relevance of myosteotosis in cachexia in more detail. Previous studies have shown fat deposition in muscle is increased in cachexia (161). But does it move from visceral or subcutaneous areas to within muscle? Which wastes first fat or muscle? Both of these questions require more investigation.

Should cut points for CT-derived low muscularity be more specific and include alterations for patients age as well as BMI? To answer this question fully the same study needs to be repeated on a much larger scale and with patients from many different ethnic backgrounds and cancer types. As well as this a baseline cut off needs to be derived for the normal, healthy population. It is also important to consider whether CT based assessment of muscle and adipose wasting should become part of the routine screening process for patients with cancer. As has been shown there is currently no way to easily differentiate between patients with cachexia, sarcopenia and malnutrition. The use of routine CT for staging cancer patients may provide an easy method of pre-operative risk stratification and allow the instigation of appropriate management or pre-habilitation before major surgery in order to improve outcome. Given also that large variations in muscularity and adiposity were seen based on age and sex this may have affected outcomes in chapters 5-7. These studies were not controlled for either and so the morphological changes seen could well differ if these factors were taken into account.

Whilst the morphology of the NMJ was investigated in great detail, the study did not comprise any assessment of proteins or function or whether the morphology of the NMJ changes in end stage cachexia. This could be investigated further by including end stage cancer patients (although the acquisition of muscle biopsies in this group is difficult) and by making an assessment of muscle function using electromyography.

The findings from this thesis may lead on to help in the clinical management of cachexia. It has helped determine criteria or highlight areas in which more work could be done to identify those patients who are suitable to enter cachexia clinical trials. Notably there could be improvements made in the way patients are screened for inclusion either using a more adequately designed screening tool or through the use of serial CT assessments. Potential biomarkers including Intelectin-1 and those seen in the metabolomics study could be useful inclusion criteria in clinical trials and may aid in monitoring the response to treatment. The finding of stability of the NMJ means that neural stimulation may be a useful novel therapeutic intervention.

8.3 Final conclusions

In conclusion this thesis has shown that direct measures of muscularity are required to assess cancer associated muscle wasting as it's difficult to infer from current screening tools or anthropometric measures such as BMI. Cut points for use in doing so however need to be age, gender and potentially disease specific. The absence of NMJ pathology in patients with cancer in contrast to animal models demonstrated that intrinsic changes in skeletal muscle demonstrate the primary locus of pathology and that future trials should consider neural stimulation as a method of increasing muscle mass. Visceral adipose tissue has been shown several times to be of potential great

importance and in particular intelectin-1 warrants further investigation through future mechanistic studies. Future research importantly should have a patient based approach as it is clear from this thesis that not all findings in animal models are translatable to patients.

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APPENDIX

Appendix 1 Patient information sheet and consent form

Biochemical and functional biomarkers of cachexia: Information for cancer patients

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.

- Part 1 tells you the purpose of this study and what will happen to you if you take part.
- Part 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Part 1

What is the purpose of the study?

The purpose of this research study is to find out what causes people who have cancer to suffer muscle wasting, lose weight and feel tired. This wasting is a significant problem for patients as it causes a reduction in both quality and quantity of life. In order to find out what causes this problem and if there is any way to predict its development, we are asking patients about to undergo surgery for cancer if they would undergo a variety of tests before, during, and after their operation. These tests are mainly to measure changes in the size and function of the patients' muscle and fat, and to see whether these changes can be predicted. In particular, we would like to take small samples of muscle, tumour, fat, blood, and urine during the patients' operation to see if there are

any biochemical changes. We aim to follow up the patients by asking for more blood and urine, as well as a needle test of the thigh muscle at clinic appointments up to 12 months after the operation. These tests are designed to cause the minimum of discomfort or inconvenience for anyone involved.

In order to find out whether the changes we see in these tests are purely related to cancer, we are also asking patients who do not have cancer, but are having surgery for other reasons, to join the study as a control group for comparison. The control group will take part in the some, but not all, of the tests undertaken by the cancer patients.

Why have I been invited?

You have been invited because you will soon be having surgery for cancer. We aim to recruit 100 participants in total. Patients undergoing purely keyhole (laparoscopic) surgery are not being invited for technical reasons.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form by one of the research team. You will still be free to withdraw at any time in the future and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care that you receive.

What will happen to me if I take part?

If you take part, you will be asked to undergo certain tests before your operation, during your operation, and at 2-3 appointments after the operation, for up to 12 months. The study appointments before and after your operation will be timed to coincide with your normal clinic appointments. Therefore, no extra visits to hospital are required if you choose to take part in this study. We anticipate the research visits will add no more than 15 minutes to your appointment. However, if for any reason we are unable to coincide your study appointments with your normal clinic appointments, we will provide taxi

transport to and from the Royal Infirmary of Edinburgh's Clinical Research Facility. The tests will include:

- ***A blood test:*** The amount of blood taken is approximately the same as one teaspoonful (5ml), and it will be taken from your arm in the usual fashion.

- ***A urine test:*** This will be performed in the usual fashion. The amount of urine taken is 20-30ml.

- ***Tests of normal activity:*** We would like to see how much activity you do during a normal week. To help assess this, we may ask you to wear a small, light physical activity meter on your thigh, under a waterproof dressing, for a week at a time. This does not inconvenience most patients, and they quickly forget that they are wearing it. These monitors can be returned by post in a pre-paid envelope you will be given.

- ***Test of functional ability (timed up-and-go and 6 minute walk):*** This test will measure your ability to get up out of a chair and walk a few metres then turn around and go back to your chair. It will only take a few minutes. The 6 minute walk test measures how far you can walk in 6 minutes. This will be done over a measured course, and should not be tiring. It can be stopped at any time if necessary.

- ***A questionnaire:*** This will take approximately 15 minutes to complete but can be taken home to do. We can provide a stamped-addressed envelope to post it to us if you take it home.

- ***CT scan review:*** We also ask that you give us permission to review the CT scans that you will have had performed as part of your routine care. This review is simply to assess the amounts of muscle and fat in your body, and does not

influence your treatment in any way. The review will be performed by a member of the research team.

We would initially plan to carry out all of the above tests at one appointment prior to the date of your operation. If, after carrying them out, you found that these tests were acceptable to perform (as we would hope), we would ask you to repeat the same tests, including the blood and urine samples, at the usual follow-up clinic appointments after your surgery for a period of up to 12 months.

If at one of the appointments after your operation you are still willing to participate, we would also ask that you give us permission to take a thigh muscle biopsy at that time. This will be performed under local anaesthetic injection but will otherwise be the same as the one that you had during your operation. This will involve a local anaesthetic injection, a small scar of 2-3 millimetres (which will be closed with paper stitches), and may leave you with a bruised feeling for a day or so. We will re-confirm your permission to perform this test at that time.

In addition to the tests described above, we will also ask permission to take tissue samples during your operation, whilst you are asleep (under general anaesthetic):

- ***A muscle biopsy:*** Whilst you are asleep (under general anaesthetic) during your operation, small muscle samples will be removed from both your abdominal wall and your thigh. The abdominal muscle sample will be pea-sized and will be taken from the incision through which your operation is being performed. In some cases we would also like to take a longer section of muscle, which will also be taken from the incision through which your operation is being performed. In patients who are undergoing operations for oesophageal cancer we will also take a small sample of muscle from your chest wall. This will not complicate your operation. The amount of muscle removed from the front of your thigh is even smaller and is taken via a tiny

incision (a few millimeters long) through which a needle is introduced. This incision will be closed by paper stitches and will leave a small mark which will fade. If you consent to have a thigh muscle biopsy in the outpatient clinic, this will be done under local anaesthetic.

- ***A fat biopsy*:** When you are asleep under general anaesthetic, a small piece of fat (again, about the size of a pea) will be taken from just under the skin and also from the fat layer inside of your abdomen.

- ***Blood and urine tests:*** If not taken in clinic before your operation, these could be taken whilst asleep under general anaesthetic.

- ***A tumour biopsy:*** We will ask you permission for a sample of tumour to be taken from the specimen removed at your operation. This will be done after the tumour has been removed.

- ***Photography:*** We would like to take a picture of the muscle used in the study. There will not be photos taken of anything else.

What do I have to do?

Apart from attendance at the appointments before your operation, and after you operation, no other responsibilities are required from your participation. We would like your permission to monitor your case for up to 5 years after your operation to see how things are going.

What are the possible disadvantages and risks of taking part?

We have taken every step in the design of this study to minimise any possible disadvantages and risks.

The tissue samples taken during your operation may cause a slightly increased risk of bleeding. This risk is very small, and the nature of the samples, together with the samples being taken by the operating surgeon, means the risk is very small indeed.

Regarding the thigh muscle biopsy, you may experience some mild discomfort and stiffness in the leg following the procedure, but this should wear off after a few hours. Also, approximately 1 in 200 people suffer with bruising following the procedure. This has been described as similar to a “dead leg” but fades in 1-2 days.

If you have are having a repeat thigh muscle biopsy this will be performed under a local anaesthetic injection. The anaesthetic agent may sting for a few seconds as it is being administered.

If you have any problems after the needle test, please contact the research team directly using the number at the end of this sheet.

What are the possible benefits of taking part?

We cannot promise that the study will help you directly, but the information we receive might help improve the treatment of patients with cancer and cancer-associated weight loss.

What happens when the research study stops?

Following your appointment after your operation, no further appointments are required. However, if any of your blood, muscle, or urine samples remain, we would ask your permission to store these samples (in anonymised form) in the University of Edinburgh so that we can consider them for use in future research studies that we may carry out (if a local Ethics Committee deems the studies appropriate).

<u>Part 2</u>

What if relevant new information becomes available?

If any new treatment for cancer or cancer-associated wasting becomes available during the time of the study, it will not be withheld from you because of your participation in this study should you need it. Furthermore, if you require any treatment for cancer or other conditions during the course of the study

(e.g. chemotherapy or radiotherapy), it will not be withheld from you because of your participation in this study.

What will happen if I don't want to carry on with the study?

You can withdraw from the study at any time. However, we would ask your permission to keep in contact with you to monitor your progress. In this way, any information that was collected during the time of your participation in the study may still be used for research purposes. Any stored blood or tissue samples that can still be identified as yours will be destroyed if you wish.

If for any reason you become unable to make decisions regarding your health, we would stop collecting information and samples from you, and we would not invite you to any more appointments. We would ask to keep and use any samples we had already collected.

What if there is a problem?

- **Complaints:** If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your question. If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.

  If you wish to make a complaint about the study, please contact NHS Lothian:

  Patient experience team
  2-4 Waterloo Place
  Edinburgh
  EH1 3EG

  Email: feedback@nhslothian.scot.nhs.uk
  Tel: 0131 536 3370

- **Harm:** In the unlikely event that something goes wrong and you are harmed during the research and this is due to someone's negligence then you may have grounds for a legal action for compensation against NHS Lothian but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you (if appropriate).

Will my taking part in this study be kept confidential?

Yes. All information which is collected about you during the course of the research will be kept strictly confidential. However, we would like to inform your GP of your involvement in this study but we will require your permission to do this. All other information about you which leaves the Royal Infirmary of Edinburgh will have your name and address removed so that you cannot be recognised from it.

If any information comes to light during the study which may have a bearing on your care, we would aim to inform the team looking after you, and with your consent we would also inform your GP.

Muscle, blood, fat and urine samples collected during the study may be transferred for the purpose of analysis to associated researchers within and outside the European Economic Area, including to commercial companies such as Novartis (a pharmaceutical company). All samples will be anonymised prior to sending and therefore you will not be identifiable. However, there will be an available link from your samples to your records so we can match any changes in your case (or your withdrawal) to your samples.

What will happen to any samples I give?

A portion of the blood samples will be immediately analysed by the Department of Biochemistry at the Royal Infirmary of Edinburgh. The remainder of the blood samples, along with the muscle, fat, and urine samples, will be transferred to the University of Edinburgh for analysis. The only individuals who will have direct access to these samples will be the members of the

research team behind this study. The samples will be analysed in the University of Edinburgh by various biochemical techniques in order to measure the levels of certain 'markers' of wasting within the various tissues.

To make sure all information remains confidential, all personal identifiers on your sample will be removed and replaced with a code. Your personal and medical history data will be stored separately and will not be processed. The key that links your data to the code on the sample will be stored in a secure location to which only authorized personnel of the SCHOOL of CLINICAL SCIENCES will have access. This is a "linked-anonymised" system. Your coded samples may also be transferred for analysis to third parties in or outside the UK. This can include collaborating academic institutions or pharmaceutical companies, which contribute to this research project.

Following all of these different analyses, if any of the samples remain, we would ask your permission to store these samples long-term (in anonymised form) in the University of Edinburgh so that we can consider them for future research studies (if a local Ethics Committee deems the studies appropriate). Professor Kenneth Fearon, Professor of Surgical Oncology, will act as custodian for any stored samples. The only other individuals who will have direct access to the stored samples will be the members of the research team behind the current study.

Will any genetic tests be done on the samples that I give?

We have no plans to perform genetic analysis within the remit of this current study. However, following this current study, we would ask your permission to store any remaining samples so that we may consider them for use in future research studies that we may carry out (if a local Ethics Committee deems the study appropriate). Future studies could potentially involve genetic analysis, but such studies are at a very early stage of planning and not yet in progress. Any results from future genetic studies will not have any healthcare

implications for you and hence we would not normally feed these results back to you.

What will happen to the results of the current research study?

The results of this study will be published in medical journals, reports and textbooks. Results will be made available to study participants through the Cancer Research UK website. You will not be identifiable in any report/publication or report unless you have specifically consented to release such information.

Who is organising and funding the research?

The research is being organised and sponsored by the University of Edinburgh. The research is being funded by Novartis.

Who has reviewed the study?

This study was given a favourable ethical opinion for conduct in the NHS by the South East Scotland Research Ethics Committee. NHS Lothian Management Approval has been obtained, and this study has also been reviewed by members of the scientific committee of Novartis.

Contact details

You may contact me (the main researcher) directly by telephoning 0131 242 6520 for further information at any time. Alternatively, if you wish to discuss this research study with someone independent of the research team, you can contact Ewan Harrison in the Department of Surgery, who is acting as an independent advisor – contact 0131 242 3615.

Many thanks for your time.

Mr Richard Skipworth
Consultant Surgeon
Department of Surgery
Royal Infirmary of Edinburgh

Patient details

Biochemical and functional biomarkers of cachexia in cancer patients

Please initial

1. I agree to take part in the above-titled study. ☐

2. I confirm that I have read and understand the information sheet **version 3 dated 25/09/2017** for the above study. I have had the opportunity to consider the information and ask questions, and I have had these answered satisfactorily. ☐

3. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

4. I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by individuals from the Sponsor [University of Edinburgh and NHS Lothian], from the NHS organisation or other authorities, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

5. I agree to my GP being informed of my participation in the study. ☐

6. I agree to my GP being informed of any information found during the study which may have a bearing on my care ☐

7. I agree to the storage of anonymised samples taken during the course of this study so that they may be considered for use in future research studies (pending a favourable ethical opinion by Lothian Local Research Ethics Committee). ☐

8. I agree to the use of samples taken during the course of this study in possible future genetic studies (pending a favourable ethical opinion by Lothian Local Research Ethics Committee). *(Optional. Only initial if you agree)* ☐

9. I understand that my anonymised samples and anonymised data may be distributed to third parties such as academic institutions or pharmaceutical companies in or outside of the UK who participate in the project ☐

10. I understand that the results of this study may be used for future commercial development of products/tests/treatments and I will not benefit financially from this ☐

11. I agree to the photographing of the muscle used in this study. ☐

_____	_____	_____
Name of patient	Date	Signature
_____	_____	_____
Person taking consent	Date	Signature

Appendix 2 QoL questionnaires

ENGLISH

EORTC QLQ-C30 (version 3)

We are interested in some things about you and your health. Please answer all of the questions yourself by circling the number that best applies to you. There are no "right" or "wrong" answers. The information that you provide will remain strictly confidential.

Please fill in your initials:
Your birthdate (Day, Month, Year):
Today's date (Day, Month, Year): 31

	Not at All	A Little	Quite a Bit	Very Much
1. Do you have any trouble doing strenuous activities, like carrying a heavy shopping bag or a suitcase?	1	2	3	4
2. Do you have any trouble taking a long walk?	1	2	3	4
3. Do you have any trouble taking a short walk outside of the house?	1	2	3	4
4. Do you need to stay in bed or a chair during the day?	1	2	3	4
5. Do you need help with eating, dressing, washing yourself or using the toilet?	1	2	3	4

During the past week:	Not at All	A Little	Quite a Bit	Very Much
6. Were you limited in doing either your work or other daily activities?	1	2	3	4
7. Were you limited in pursuing your hobbies or other leisure time activities?	1	2	3	4
8. Were you short of breath?	1	2	3	4
9. Have you had pain?	1	2	3	4
10. Did you need to rest?	1	2	3	4
11. Have you had trouble sleeping?	1	2	3	4
12. Have you felt weak?	1	2	3	4
13. Have you lacked appetite?	1	2	3	4
14. Have you felt nauseated?	1	2	3	4
15. Have you vomited?	1	2	3	4
16. Have you been constipated?	1	2	3	4

Please go on to the next page

During the past week:	Not at All	A Little	Quite a Bit	Very Much
17. Have you had diarrhea?	1	2	3	4
18. Were you tired?	1	2	3	4
19. Did pain interfere with your daily activities?	1	2	3	4
20. Have you had difficulty in concentrating on things, like reading a newspaper or watching television?	1	2	3	4
21. Did you feel tense?	1	2	3	4
22. Did you worry?	1	2	3	4
23. Did you feel irritable?	1	2	3	4
24. Did you feel depressed?	1	2	3	4
25. Have you had difficulty remembering things?	1	2	3	4
26. Has your physical condition or medical treatment interfered with your family life?	1	2	3	4
27. Has your physical condition or medical treatment interfered with your social activities?	1	2	3	4
28. Has your physical condition or medical treatment caused you financial difficulties?	1	2	3	4

For the following questions please circle the number between 1 and 7 that best applies to you

29. How would you rate your overall health during the past week?

1	2	3	4	5	6	7
Very poor						Excellent

30. How would you rate your overall quality of life during the past week?

1	2	3	4	5	6	7
Very poor						Excellent

ENGLISH

EORTC QLQ – OG25

Patients sometimes report that they have the following symptoms or problems. Please indicate the extent to which you have experienced these symptoms or problems during the past week. Please answer by circling the number that best applies to you.

During the past week:	Not at all	A little	Quite a bit	Very much
31. Have you had problems eating solid foods?	1	2	3	4
32. Have you had problems eating liquidised or soft foods?	1	2	3	4
33. Have you had problems drinking liquids?	1	2	3	4
34. Have you had trouble enjoying your meals?	1	2	3	4
35. Have you felt full up too quickly after beginning to eat?	1	2	3	4
36. Has it taken you a long time to complete your meals?	1	2	3	4
37. Have you had difficulty eating?	1	2	3	4
38. Have you had acid indigestion or heartburn?	1	2	3	4
39. Has acid or bile coming into your mouth been a problem?	1	2	3	4
40. Have you had discomfort when eating?	1	2	3	4
41. Have you had pain when you eat?	1	2	3	4
42. Have you had pain in your stomach area?	1	2	3	4
43. Have you had discomfort in your stomach area?	1	2	3	4
44. Have you been thinking about your illness?	1	2	3	4
45. Have you worried about your health in the future?	1	2	3	4
46. Have you had trouble with eating in front of other people?	1	2	3	4
47. Have you had a dry mouth?	1	2	3	4
48. Have you had problems with your sense of taste?	1	2	3	4
49. Have you felt physically less attractive as a result of your disease or treatment?	1	2	3	4

Please go on to the next page

During the past week:	Not at all	A little	Quite a bit	Very much
50. Have you had difficulty swallowing your saliva?	1	2	3	4
51. Have you choked when swallowing?	1	2	3	4
52. Have you coughed?	1	2	3	4
53. Have you had difficulty talking?	1	2	3	4
54. Have you worried about your weight being too low?	1	2	3	4
55. Answer this question only if you lost any hair: If so, were you upset by the loss of your hair?	1	2	3	4

By placing a tick in one box in each group below, please indicate which statements best describe your own health state today.

Mobility

I have no problems in walking about ❑

I have some problems in walking about ❑

I am confined to bed ❑

Self-Care

I have no problems with self-care ❑

I have some problems washing or dressing myself ❑

I am unable to wash or dress myself ❑

Usual Activities *(e.g. work, study, housework, family or leisure activities)*

I have no problems with performing my usual activities ❑

I have some problems with performing my usual activities ❑

I am unable to perform my usual activities ❑

Pain / Discomfort

I have no pain or discomfort ❑

I have moderate pain or discomfort ❑

I have extreme pain or discomfort ❑

Anxiety / Depression

I am not anxious or depressed ❑

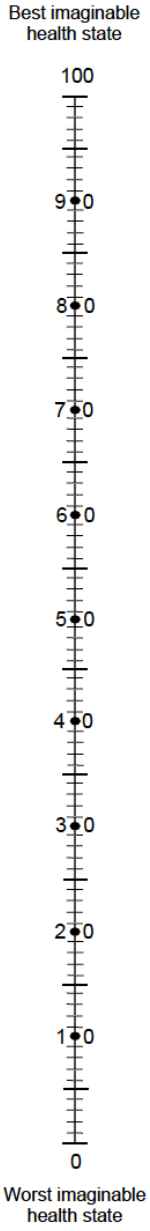
I am moderately anxious or depressed ❑

I am extremely anxious or depressed ❑

To help people say how good or bad a health state is, we have drawn a scale (rather like a thermometer) on which the best state you can imagine is marked 100 and the worst state you can imagine is marked 0.

We would like you to indicate on this scale how good or bad your own health is today, in your opinion. Please do this by drawing a line from the box below to whichever point on the scale indicates how good or bad your health state is today.

Your own health state today



Appendix 3 Published and presented work

Publications:

Miller J and Skipworth RJE. Novel molecular targets of muscle wasting in cancer patients. Current Opinion in Clinical Nutrition and Metabolic Care. 2019; 22(3):196-204

Main text: Introduction

Miller J, Laird BA, Skipworth RJE. Immunological regulation of cancer cachexia. Journal of Cancer Metastasis and Treatment 2019; 5:68. Doi: 10.20517/2394-4722.2019.001

Main text: Introduction

Miller J, Ramage MI, Skipworth RJE. New developments in targeting cancer cachexia. Systemic effects of advanced cancer. Springer Nature. Submitted 2020

Main text: Introduction

Miller J, Wells L, Nwulu U, Currow D, Johnson MJ, Skipworth RJE. Validated screening tools for the assessment of cachexia, sarcopenia and malnutrition: A systematic review. American Journal of Clinical Nutrition 2018;108:1196–1208

Main text: Chapter 1

Boehm I*, Miller J*, Wishart TM, Wigmore SJ, Skipworth RJE, Jones RA, Gillingwater TH. Neuromuscular junctions are stable in patients with cancer cachexia. J Clin Invest 2020; 130(3):1461-1465

Main text: Chapter 3 *Joint first authors

Minty G, Hoppen A, Boehm I, Alhindi A, Gibb L, Potter E, Wagner B, Miller J, Skipworth R, Gillingwater T, Jones R. aNMJ Morph – a simple macro for rapid analysis of neuromuscular junction (NMJ) morphology. Royal Society Open Medicine 2020 https://doi.org/10.1098/rsos.200128

Main text: Chapter 3

Miller J, Dreczkowski G, Ramage MI, Wigmore SJ, Gallagher IJ, Skipworth RJE. Adipose depot gene expression and Intelectin-1 in the metabolic response to cancer and cachexia. J Cachexia Sarcopenia and Muscle 2020 doi: 10.1002/jcsm.12568. [Epub ahead of print]

Main text: Chapter 4

Miller J, Alshehri A, Ramage MI, Stephens NA, Mullen AB, Boyd M, Ross JA, Wigmore SJ, Skipworth RJE. Plasma Metabolomics identifies lipid and amino acid markers of weight loss in patients with upper gastrointestinal cancer. Cancers 2019;11:1594

Main text: Chapter 5

Oral presentations:

Adipose depot specific mRNA transcriptomics in cancer cachexia: 11th International conference on cachexia, sarcopenia and muscle wasting. Maastricht, December 7th-9th 2018

Characterisations, mechanisms and clinical impact of altered body composition in upper gastrointestinal cancer. 8th International seminar of precision medicine in palliative and supportive care. Edinburgh, December 12th-14th 2018

Miller J, Boehm I, Wishart TM, Wigmore SJ, Jones RA, Gillingwater TH Skipworth RJE. Neuromuscular junctions are stable in patients with cancer cachexia. Edinburgh school of surgery day 22nd Nov 2019.
Winner of MacLeod medal for best clinical presentation

Validated screening tools for the assessment of cachexia, sarcopenia and malnutrition: A systematic review. Miller J, Wells L, Nwulu U, Currow D, Johnson MJ, Skipworth RJE. Edinburgh school of surgery day 22nd Nov 2019.
Winner of the Professor Ken Fearon Memorial Medal

Poster presentations:

Miller J, Alsheri A, Stephens NA, Ramage MI, Wigmore SJ, Ross JA, Watson DG, Skipworth RJE. Plasma metabolomic analysis of weight loss in upper gastrointestinal cancer patients undergoing surgical resection. 4th Cancer Cachexia Conference. Philadelphia, September 14th-16th 2018

Miller J, Ramage MI, Wigmore SJ, Ross JA, Gallagher IJ, Skipworth RJE. Adipose depot gene expression in oesophago-gastric cancer.

Miller J, Ramage MI, Schuepbach E, Roubenof R, Deans DAC, Wigmore SJ, Ross JA, Jacobi C, Skipworth RJE. CT derived measures of muscle mass in upper gastrointestinal cancer patients: Prevalence and relationship with quality of life and muscle function

Published abstracts:

Miller J, Ramage MI, Wigmore SJ, Ross JA, Gallagher IJ, Skipworth RJE. Adipose depot specific mRNA transcriptomics in cancer cachexia. Journal of Cachexia, Sarcopenia and Muscle. 2018; 9(6):1151

Miller J, Alsheri A, Stephens NA, Ramage MI, Wigmore SJ, Ross JA, Watson DG, Skipworth RJE. Plasma metabolomics in oesophago-gastric cancer: A pilot study. Journal of Cachexia, Sarcopenia and Muscle. 2018; 9(6):1161

Miller J, Ramage MI, Schuepbach E, Roubenof R, Deans DAC, Wigmore SJ, Ross JA, Jacobi C, Skipworth RJE. CT derived measures of muscle mass in upper gastrointestinal cancer patients: Prevalence and relationship with quality of life and muscle function. British Journal of Surgery 2018; 105(s6):26

Miller J, Alsheri A, Stephens NA, Ramage MI, Wigmore SJ, Ross JA, Watson DG, Skipworth RJE. Plasma metabolomic analysis of weight loss in upper gastrointestinal cancer patients undergoing surgical resection. British Journal of Surgery 2018; 105(s6):37

Miller J, Ramage MI, Wigmore SJ, Ross JA, Gallagher IJ, Skipworth RJE. Adipose depot gene expression in oesophago-gastric cancer. British Journal of Surgery 2018; 105(s6):37

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REVIEW

Novel molecular targets of muscle wasting in cancer patients

Janice Miller and Richard J.E. Skipworth

Purpose of review
Cancer-associated muscle wasting affects many patients and leads to reduced patient function, decreased quality of life and poor responses to surgical and oncological treatments. Despite advancements in the understanding of its pathophysiology, no current treatment or accepted strategy for successful management exists. In this review, we provide an update on potential novel therapeutic targets in cancer cachexia.

Recent findings
Recent research has focused on molecular mechanisms underlying cancer-associated muscle wasting, allowing identification of potential therapeutic targets and the development of several promising drugs. However, due to the multifactorial and patient-specific pathogenesis of cachexia, the demonstration of a measurable and meaningful clinical effect in randomized controlled trials has proven difficult. Potential novel targets such as circulating macrophage inhibitory cytokine 1/growth differentiation factor 15 and ZRT/IRT-like protein 14 have shown relevance in animal models, but their therapeutic manipulation has yet to be translated to patients. Increasing evidence has suggested that a single therapy may not be successful and a targeted, multimodal approach is required.

Summary
The management of cancer-associated muscle wasting is complex. Future clinical trials should focus on early multimodal therapeutic interventions involving targeted therapies, with careful deliberation of chosen nutritional and functional outcomes.

Keywords
cachexia, cancer, muscle wasting, therapeutics

INTRODUCTION

In contrast to the consistent development of new anticancer therapies, there remains no approved pharmaceutical treatment for muscle wasting in cancer. Muscle wasting is a key component of the cancer cachexia syndrome – defined as 'an ongoing loss of skeletal muscle mass (with or without loss of fat mass) that cannot be fully reversed by conventional nutritional support and leads to progressive functional impairment' [1]. Cachexia affects 30–70% of patients with cancer with the greatest incidence of weight loss seen amongst patients with solid tumours. Although loss of muscle mass is the defining feature of cancer cachexia, differences in the underlying mechanisms have been reported between experimental models and human tumour types [2]. This diversity leads to challenges in the development of effective therapeutic management strategies, as well as difficulties in the design of clinical trials.

Muscle 'recovery' is a complex notion, which could be interpreted as the amelioration of wasting, an increase in muscle mass, and/or an improvement in functional outcomes. The failure of most cachexia-related clinical trials has been due to an inability to show improvement in muscle function. This may be because the treatment is not effective in this respect but may also be due to a lack of consensus regarding the best measure of function, leading to differing outcome measures being used across various clinical trials. To date most trials have been conducted on palliative patients with advanced disease and this may be why many drugs have been deemed ineffective. Despite this, promising trials are currently ongoing with early results suggesting that single therapies to treat muscle wasting maybe

Department of Clinical Surgery, University of Edinburgh, Royal Infirmary of Edinburgh, Edinburgh, UK

Correspondence to Richard J.E. Skipworth, School of Clinical Sciences, University of Edinburgh, 49 Little France Crescent, Edinburgh EH16 4SB, UK. Tel: +44 0 131 2423176;
e-mail: richard.skipworth@nhslothian.scot.nhs.uk

Curr Opin Clin Nutr Metab Care 2019, 22:000–000
DOI:10.1097/MCO.0000000000000555

KEY POINTS

- Patient-centred research is important to ensure the translation of promising therapeutic targets in animal models.
- Specific novel targets include circulating and muscle-specific mediators, as well as those involved in tissue cross-talk including IL-6, macrophage inhibitory cytokine 1/growth differentiation factor 15, tumour necrosis factor-related weak inducer of apoptosis/factor-inducible 14 and ZRT/IRT-like protein 14.
- Muscle wasting in cancer is best targeted with a patient-specific approach complimented by multimodal therapy.

benefited by a wider multimodal approach [3**]. This review aims to summarize the most recent research into cancer cachexia, and will describe novel targets including mediators and receptors, be they circulating, muscle-specific, anticatabolic or those involved in tissue cross talk. We will focus specifically on those targets that have already been shown to be important in humans. A summary of those that will be discussed throughout the review is provided in Fig. 1. This figure demonstrates the complex interplay between mediators of muscle wasting, and their resulting effect on end organs.

Circulating mediators

There are many circulating mediators that have systemic effects in the pathogenesis of cachexia;

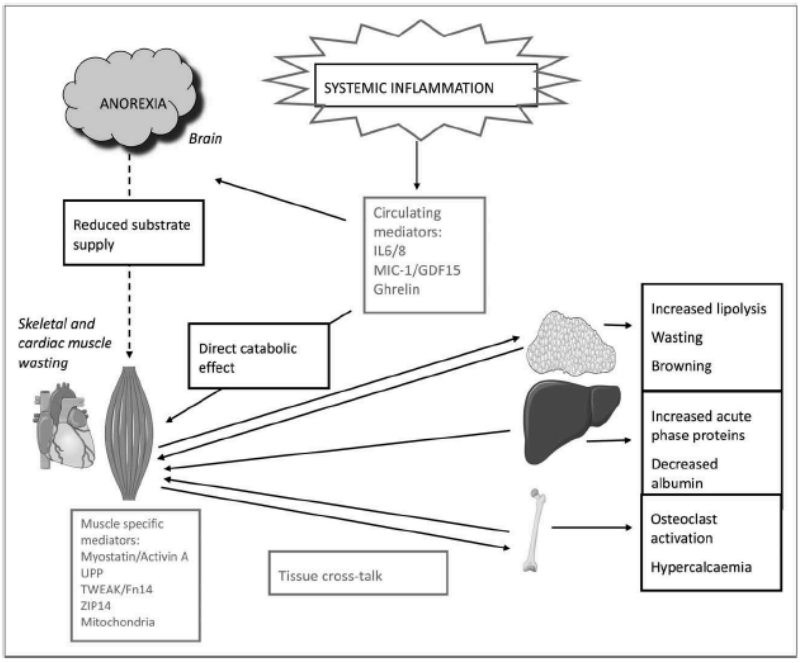

FIGURE 1. Novel mediators of cancer-associated muscle wasting. Tumour and host-derived circulating mediators have been shown to act on target tissues to stimulate excess catabolism or promote central anorectic effects. Numerous proinflammatory cytokines, mRNA and microvesicles are generated through tumour and tissue cross-talk and many mediators have autocrine and paracrine effects on muscle itself.

of these, the interleukins and, in particular, IL-6 have been the most widely researched. As different mechanisms of action of IL-6 continue to be discovered, it continues to lend itself as a novel mediator.

Interleukins

IL-6 is a driver of systemic inflammation with levels correlating with weight loss and reduced survival in cancer patients [4,5]. IL-6 signals through the membrane-bound receptor glycoprotein 130 found in most tissues in the body [6]. Once bound it activates Janus kinase tyrosine kinase leading to phosphorylation of tyrosines and the binding of signal transducer and activator of transcription (STAT) proteins [7]. STAT proteins can translocate to the nucleus and increase the transcription of genes involved in immune function, cell proliferation, differentiation and apoptosis [7]. IL-6 administration in humans without cancer is capable of inducing muscle wasting, and deletion of the IL-6 gene in the $APC^{Min/+}$ mouse (colorectal cancer model that develops IL-6 dependent cachexia) prevented the development of cachexia [4]. IL-6 when secreted by tumour cells can also increase autophagy in myotubes when joined with soluble IL-6 receptor – so called trans-signalling [6]. Increasing autophagy and IL-6 levels have been associated with poor prognosis and weight loss in lung cancer patients [6]. IL-6 trans-signalling has also shown to be involved in cross-talk between tumour, muscle and adipose tissue in genetic mouse models of pancreatic cancer cachexia. In such models, tumour-derived IL-6 is shown to result in skeletal muscle soluble IL-6R production, increasing lipolysis via IL-6 trans-signalling, and further increasing muscle wasting [7,8].

Previous trials of anti-IL6 antibody (ALD 518) in small numbers of patients with nonsmall-cell lung cancer showed reduced loss of lean body mass (LBM) compared with controls although this finding was NS [9]. Tocilizumab, another anti-IL6 receptor antibody, was investigated in rheumatoid arthritis patients and was shown to provide a significant gain in LBM and skeletal muscle mass index following 6–12 months of treatment [10]. This study did not assess effects on muscle function and previous studies have raised concerns about its cardiometabolic safety, leaving us unable to conclude its real benefit for patients. There have been no trials in cancer patients.

Another proinflammatory cytokine that has been implicated in the pathogenesis of cachexia is IL-8. It is a member of the chemokine family and strongly attracts motile neutrophils and lymphocytes, aiding angiogenesis and tumour development. Serum levels of IL-8 are raised in many different cancer types and most notably have been associated with poorer survival rates in cachectic pancreatic cancer patients [11,12]. There are currently no ongoing trials assessing IL-8 in cachexia.

Macrophage inhibitory cytokine 1/growth differentiation factor 15

Macrophage inhibitory cytokine-1/growth differentiation factor 15 (MIC-1/GDF15) is associated with many metabolic disease processes including obesity and anorexia, and has consistently been shown to induce cachexia in animal models [13]. The link between MIC-1/GDF15 in humans was first suggested on the basis that increased levels were associated with weight loss in prostate cancer patients [14]. MIC-1/GDF15 acts on the hypothalamus and brainstem to cause anorexia, leading to drastic weight loss and cachexia. Ablation of MIC-1/GDF15 in animal models resulted in increased body weight, whereas overexpression led to lower body weight and fat mass [13]. The exact mechanisms by which MIC-1/GDF15 acts are relatively unclear. Several studies have identified GFRAL, a member of the glial-derived neurotrophic factor receptor α family, as the receptor by which MIC-1/GDF15 binds. It is also thought to be affected by growth differentiation factor 11 (GDF11), which is closely related to myostatin and is a more potent ligand for the activin type II (ActRII/Alk) complex than myostatin (discussed further below) [15•,16]. Raised levels have been shown to cause cachexia in mice and also to raise circulating levels of activin A leading to muscle wasting. Increased levels of GDF11 cause upregulation of GDF15 and the recruitment of SMAD2/3. Inhibition of GDF15 was shown to improve appetite but did not reverse the GDF11-induced muscle wasting which was reversed by antibodies to the activin type II receptor (ActRIIB) [17]. Human trials of anti MIC-1/GDF15 are awaited.

Muscle-specific mediators

There are many mediators of pathways leading to muscle wasting, most of which are involved in protein degradation. Some act directly within or on muscle cells whilst others act as local paracrine mediators.

Myostatin and activin A

A recent factor that has been the subject of much research is a member of the transforming growth factor beta (TGFβ) family – myostatin. Animals and humans who have nonfunctional copies of the myostatin gene have been shown to demonstrate dramatic muscle hypertrophy [18]. Skeletal muscle

is the main secretory source of myostatin. It activates Smad2 and 3 transcription factor complexes through ActRIIB. Enhanced production of myostatin in mice leads to muscle atrophy, whereas genetic inhibition leads to enhanced muscle mass and fibre size [18]. The exact mechanisms via which myostatin leads to muscle loss are unclear but it is thought to be due to its ability to inhibit protein kinase B (Akt) and therefore the target of rapamycin complex 1 pathways. This then enhances protein synthesis as well as its ability to inhibit myosatellite cell proliferation [18]. In addition to myostatin, other TGFβ family members such as Activin A are upregulated in skeletal muscle [19]. Activin A is increased following activation of the TNFα/transforming growth factor beta-activated kinase-1 signalling pathway. Many cancers lead to altered expression of activin A and are capable of inducing expression in muscle [20]. Due to the complex interactions within these pathways the blocking of one target alone may not be sufficient enough to prevent muscle wasting. The ActRIIB receptor is common to both myostatin and the activins and its blockade in the rodent C26 model of cancer cachexia was shown to be effective in reversing muscle wasting and also prolonging survival [21].

Much attention has been given to therapeutic agents that target myostatin and the ActRIIB pathway in humans. The human derivative of the myostatin neutralizing mouse IgG1 mAb Landogrozumab (LY2495655) was investigated in a phase 2 trial in palliative pancreatic cancer patients with stage II–IV disease [22▪]. However, there was an observed trend towards decreased overall survival in the treatment groups for reasons that are unclear. Although there was no significant difference in performance status on treatment, patients with less than 5% weight loss had better performance-related results [22▪]. This may be indicative of the fact that LY2495655 simply reduces muscle loss rather than fully reversing the cachectic process, or that patients with end stage disease and refractory cachexia were recruited. These findings are in contrast to those from a randomized trial involving 365 elderly patients who had fallen, where LY2495655 treatment increased LBM and improved functional measures of muscle power albeit nonsignificantly [23]. Initially thought to be more promising is Bimagrumab (BYM338), a human monoclonal anti-ActRII antibody. It prevents the binding of ligands which signal through this pathway to their receptor, promoting muscle hypertrophy and accelerating recovery from muscle-wasting conditions in animals. Results from recent clinical trials have been mixed with increases in muscle volume seen in patients with inclusion body myositis, sarcopenia and disuse atrophy following fracture [24–26]. However, coprimary endpoints of functionality were again not satisfied. There have been no trials in cancer patients.

Tumour necrosis factor-related weak inducer of apoptosis and factor-inducible 14

Tumour necrosis factor-related weak inducer of Apoptosis (TWEAK) and its related receptor, fibroblast growth factor-inducible 14 (Fn14), are members of the tumour necrosis factor/tumour necrosis factor receptor superfamily. Their expression is upregulated in tumours and leads to potent skeletal muscle wasting [27]. TWEAK causes myotube atrophy through coordinated activation of the ubiquitin-proteasome pathway (UPP), autophagy and caspases. Animal models have shown antibodies to Fn14 are able to extend lifespan by inhibiting tumour-induced weight loss [27]. Fn14 signalling seems to occur in the tumour itself, rather than the host, as tumours in Fn14 and TWEAK-deficient hosts developed cachexia that was comparable with that of wild type mice [27]. These animal model results suggest that Fn14 antibodies may be a promising therapeutic approach for the future.

Ubiquitin proteasome pathway and autophagy

Muscle mass in healthy individuals remains fairly constant due to a balance between synthesis and degradation. Specific molecules may be upregulated in response to certain stimuli for example members of the UPP. Forkhead box O transcription factors play a key role. They are phosphorylated and inactivated by phosphatidylinositol 3-kinase-Akt/PKB, and translocate into the cell nucleus where they induce the transcription of E3 ubiquitin ligases, muscle RING-finger protein-1 and atrogin-1/muscle atrophy F-box, as well as autophagy-related genes microtubule-associated protein 1A/1B-light chain 3 and Bnip3 [28]. Together, this large intracellular system regulates muscle mass in response to tumour factors and circulating cytokines. Inhibition of these proteolytic pathways has been investigated in clinical trials. The drug Bortezomib, a proteasome inhibitor, has not shown any effect on muscle wasting and weight loss in patients with metastatic pancreatic cancer [29], whereas MG132, a different proteasome inhibitor, improved muscle mass in animal models possibly due to a different mechanism of action to that observed in human muscle [2]. Indeed, evidence for upregulation of markers of the UPP in humans is not as robust as that seen in animals, which is perhaps why these drugs have failed in clinical trials.

Autophagy is a homeostatic mechanism that involves lysosomal dependent degradation of cellular products, including damaged organelles and proteins, and intracellular pathogens. It works in all eukaryotic cells as a quality control mechanism preventing accumulation of degenerated proteins. Conditions of stress or starvation induce its upregulation to replace damaged cellular products and mobilize energy stores. It is thought to play a role in the regulation of muscle mass, and muscle-specific deletions of autophagy genes (e.g. autophagy related 7) in mice have been shown to lead to muscle wasting [30]. Markers of increased autophagy have been demonstrated in patients with oesophageal, lung, upper gastrointestinal and pancreatic cancer patients [30]. The pathophysiological role of autophagy in cachexia however, is not yet fully understood due to difficulties in assessing the autophagic flux in the clinical setting.

ZRT/IRT-like protein 14

The metal ion transporter ZRT and IRT-like protein 14 (ZIP14) is a newly identified critical mediator of cancer cachexia in mouse models of various types of metastatic cancer. It is a member of the solute carrier 39A zinc transporter family typically expressed in the liver, which co-ordinates zinc uptake by cells [31]. It is upregulated in the muscles of mice and patients with metastatic cancer and is predominantly induced by TNFα and TGFβ. ZIP14 exerts its effect in animals by reducing the expression of MyoD and Mef2c therefore blocking muscle cell differentiation. ZIP14-mediated zinc accumulation in well differentiated muscle cells leads to loss of myosin heavy chains [32▼]. Muscle-specific deletion reduces muscle wasting in animal models of cancer cachexia [32▼]. Drugs that inhibit ZIP14 may therefore offer future promise.

Mitochondria and exercise

The targeting of mitochondrial dysfunction is a potential therapeutic opportunity to normalize energy metabolism in catabolic conditions. Exercise is an important regulator of mitochondrial function and skeletal muscle metabolism, but compliance with exercise regimens in patients with advanced cancer is difficult due to chronic fatigue and cancer related comorbidities [33]. The benefits of increased exercise include reduced adiposity, increased muscularity, lower chronic inflammation, increased muscle mitochondrial content and function through peroxisome proliferator-activated receptor gamma coactivator 1-alpha expression, and improved insulin sensitivity [34]. The exercise mimetic 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR), an adenosine monophosphate-activated protein kinase (AMPK) activator, has been investigated in C26 mice [35]. Activation of AMPK is associated with a proatrophic signal in muscle and also demonstrates anti-inflammatory effects. AICAR, the AMPK agonist, has been shown to suppress IFNγ/TNFα-induced atrophy. IFNγ/TNFα impair mitochondrial oxidative respiration in myotubes and promote a metabolic shift to aerobic glycolysis. AICAR was able to partially restore metabolic function and prevent muscle wasting in mouse models of cancer.

Mitochondrial dysfunction can also be due to disruption of fission and fusion processes. The latter is regulated by mitofusin 1 and 2 (MFN-1/2) and optic atrophy protein 1. MFN-1/2 have differing functions in regulation of guanosine triphosphate tethering and formation of fusion complexes with OPA 1 (mitochondrial dynamin like GTPase) regulating these processes. The loss of mitochondrial fusion has been shown to lead to muscle wasting in MFN-1/2 knockout mice therefore lending it as an important novel mediator in the future of investigation of cancer-associated muscle wasting [36].

Anticatabolics

Several anticatabolic mediators have been the subject of important clinical trials in cachexia. Their mechanisms of action, and controversies surrounding the drugs used to target them, are discussed below.

Ghrelin

Ghrelin is an orexigenic gut hormone primarily secreted by the endocrine cells of the gastric mucosa. It is a 28 amino acid peptide that is an endogenous ligand for the growth hormone (GH) secretagogue receptor, and serum levels have been found to be elevated in cancer patients with muscle wasting [37]. Ghrelin can increase body weight by increasing appetite, GH secretion and prevention of muscle catabolism [38]. It has anti-inflammatory actions through its ability to inhibit TNFα, C-reactive protein, IL-1β and IL-6 in mouse models. Ghrelin is also able to initiate its orexigenic effect by stimulation of the hypothalamus and brain stem neurons, and can increase the expression of cannabinoid-1 and major histocompatibility complex 1 receptors on vagal afferents [39]. The effect on GH occurs during conditions of starvation. Ghrelin stimulates GH to spare protein stores at the expense of fats therefore demonstrating GH-dependent and independent anticachectic effects [40].

The potential of Ghrelin as a treatment for cancer cachexia has been investigated in the double-blind, placebo-controlled ROMANA I and

II trials (703 patients), and the ROMANA 3 (513 patients) safety extension study [41,42▪]. Anamorelin, an oral and specific ghrelin receptor agonist, was given to patients with advanced nonsmall-cell lung cancer and cachexia. LBM increased in patients taking Anamorelin compared with placebo in all trials, as was body weight and anorexia-cachexia related symptoms. However, the trials were unable to demonstrate differences in handgrip strength [39]. Food intake was not recorded and it is therefore unclear as to whether an increase in appetite and nutritional status led directly to the observed changes in body mass. Besides anamorelin, the other novel Ghrelin agent macimorelin (NCT01614990) is currently under investigation in patients with palliative cancer, the results of which are expected in 2020 [43].

Selective androgen receptor modulators

Other emerging anabolic agents are the selective androgen receptor modulators. These drugs, most notably Enobosarm, which was investigated in the POWER 1 and 2 trials, are a new class of nonsteroidal drugs [44,45]. They are unique in their ability to increase muscle mass without the usual anabolic side effects. The POWER trials were randomized, double blinded, placebo controlled in patients with advanced lung cancer who had at least 2% weight loss in the previous 2 months. They both involved 3 mg of enobosarm or placebo daily at the start of chemotherapy (Platinum + Taxane in POWER 1 and Platinum + Non-Taxane in POWER 2). Unpublished results have shown a significant increase in LBM but no increase in mean stair climb power [45].

Tissue cross-talk

The importance of tissue cross-talk in cachexia research is gaining more attention, and in particular, fat-muscle cross-talk and circulating microRNA mediators of systemic cross talk. Effects on cardiac function lead to wasting and decreased innervation, possibly causing death [46].

Fat-muscle cross-talk

Genetic ablation of lipolytic pathways in adipocytes has been shown to protect against muscle mass loss in preclinical models of cancer cachexia [46]. In particular, inhibition of lipolysis through genetic ablation of adipose triglyceride lipase or hormone sensitive lipase reduces muscle wasting [47]. Potential mediators of fat-muscle crosstalk include myokines, adipokines or free fatty acids [48]. With regard to human findings, previous studies have described worsening myosteatosis in association with increasing degrees of cachexia, suggesting a direct link between muscle and fat metabolism [49]. These findings support the loss of visceral fat driven by lipolytic mechanisms as an early event in cancer cachexia resulting in increased fat deposits in skeletal muscle.

Therapeutic manipulation of lipid metabolism has shown some promise in the amelioration of muscle wasting. For example, inhibition of fatty acid oxidation with Etoxomir (an inhibitor of carnotine palmitoyltransferase-1) has been shown to stop muscle wasting in animal models [50,51]. Parathyroid hormone and parathyroid hormone-related peptide (PTHrP) is thought to cause adipose tissue browning and increased energy production via uncoupling protein 1 through activation of protein kinase A. It is also thought to contribute to muscle wasting through activation of the UPP [52]. Treatment with an antibody to neutralise PTHrP prevented muscle wasting, as well as adipose tissue browning and loss, in mice bearing the Lewis Lung Carcinoma [53].

Involvement of other tissues

Many other organs or tissues are involved in cancer cachexia, and cross-talk between such organs may be facilitated by systemic inflammation. The liver is a major contributor to systemic inflammation via the synthesis of acute phase proteins. The brain induces changes in neuropeptides that regulate appetite, and activation of the hypothalamic pituitary axis leads to the release of glucocorticoids that act to promote muscle wasting [46]. Loss of muscle is associated with increased osteoclast function resulting in increased bone resorption and worsened function and quality of life [46].

Microvesicles and microRNAs

The study of exosomal micro RNAs (miRNAs) and muscle-specific miRNAs (myomiRs) in muscle wasting is a relatively new field. Both are thought to play a role in the transduction of inflammatory signals and the activation of the catabolic status in muscles [54▪]. miRNAs are embedded in microvesicles, which are small membrane-derived particles that are potentially able to communicate with other cells in the body by fusing with their plasma membrane, thus allowing the delivery of various molecules including miRNAs, mRNA and proteins [55]. High levels of tumour-released extracellular heat shock protein 70 and 90 which are bound inside microvesivles have been detected in the serum of cachectic mice [56].

Exosomal-transported miRNAs are of particular interest as they are involved in the development and

continuation of inflammation in cachexia. Changes in the expression profile of miRNAs may indicate the regulation of protein synthesis and degradation pathways in skeletal muscle and thus indicate the presence and or progression of muscle wasting [55]. One of the miRNAs whose concentration is increased in cancer patients is miR-21 [57]. It is thought to be secreted from the tumour, transported in the blood stream, and able to induce the apoptosis of muscle cells via its ability to bind and activate Toll-like receptor 7 (TLR-7) in rats and TLR-8 in human myoblasts. miR-21 thereby promotes apoptosis through the activation of the c-Jun N-terminal kinase pathway [58]. A recent study has demonstrated that IMO-8503, a TLR7/8/9 antagonist, is able to inhibit cell death caused by circulating miRNAs in tumour-bearing mice [59]. Circulating mediators of cachexia and inflammation such as miRNAs offer avenues not only for therapeutic intervention, but also biomarker development.

Current challenges in cachexia management

The complex pathogenesis of cachexia combined with differences in host genotype pose many difficulties in cachexia management. It is becoming more apparent that treatment with a single agent may not be possible and we therefore need to consider the following issues.

The need for multimodal therapy

The development of therapy for muscle wasting in cancer has focused on improving appetite, reducing inflammation and modifying muscle protein metabolism. However, no single agent has yet been shown to be able to achieve all of these aims, highlighting the need for a multimodal approach. This concept has led to the development of the MENAC trials (Multimodal-Exercise, Nutrition and Anti-inflammatory medication for Cachexia) for palliative lung and pancreatic cancer patients [3▪▪,60]. The pre-MENAC trial showed that a multimodal intervention in cachexia might improve weight, leading to progression of the phase III MENAC trial (NCT01419145) [61,62▪]. The trial therapy consists of nonsteroidal antiinflammatories; eicosapentaenoic acid (an immmunomodulatory n3 fatty acid), oral nutritional supplementation and dietary counselling; and a physical exercise programme using resistance and aerobic training versus standard cancer care [3▪▪]. The trial is ongoing. Outcome measures include change in body weight, muscle mass, quality of life and physical activity.

The need for patient-specific therapy

Based on the current body of research available it is still not possible to predict which patients will develop cachexia. Further research into genetics may reveal that some have a genetic predisposition. Candidate gene studies have shown single nucleotide polymorphisms in the P-selectin, activin A receptor type 2B, angiotensin-1 converting enzyme, leptin receptor and tumour necrosis factor genes are associated with low muscle mass in patients with cancer [63]. Sexual dimorphism is also evident, with males and females showing differences in muscle mass, fibre type and size in response to tumour stimuli [64]. Future interventions may therefore be better applied to a sex-specific or patient-specific approach. Definitive end points in cachexia trials have also been difficult to reach [65]. The relationship between muscle mass and function has been shown to be complex and unlikely to be linear [65]. The success of therapeutic trials will be heavily influenced by the choice of possible outcome measures, and so these should be considered carefully at the initial design stage [65].

CONCLUSION

Muscle wasting in cancer is multifactorial and complex. It is underpinned by hypercatabolism, a net loss of energy, and systemic inflammation. Although the effects of cachexia are most clinically obvious and physically devastating later in the disease process, detectable molecular changes will already have taken place. The administration of targeted treatment should therefore be early and prophylactic, prior to the development of significant weight loss. Such an approach will require the identification of robust biomarkers to accompany trail methodology. Many therapeutic targets to date have shown promise in preclinical models, but have not been fully efficacious in clinical trials, particularly in regard to their ability to improve muscle function as well as mass. Targeted treatments need to be delivered in a precision medicine and patient-specific approach, and supported by multimodal management regimens.

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Conflicts of interest

There are no conflicts of interest.

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Review

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The immunological regulation of cancer cachexia and its therapeutic implications

Janice Miller[1], Barry J. A. Laird[2], Richard J. E. Skipworth[1]

[1]Clinical Surgery, Royal Infirmary of Edinburgh, University of Edinburgh, Edinburgh EH16 4SA, UK.
[2]Edinburgh Palliative and Supportive Care Group, University of Edinburgh, Western General Hospital, Edinburgh EH4 2XU, UK.

Correspondence to: Dr. Richard J. E. Skipworth, Clinical Surgery, Royal Infirmary of Edinburgh, University of Edinburgh, 51 Little France Crescent, Edinburgh EH16 4SA, UK. E-mail: richard.skipworth@nhslothian.scot.nhs.uk

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Abstract

Cachexia affects the majority of patients with advanced cancer. It leads to poor surgical and oncological outcomes, and negatively affects quality of life. It has long been reported that components of the host immune system, including pro-inflammatory cytokines such as IL-1α, IL-6, TNF-α and INF-γ, participate in the syndrome of cachexia. Yet therapeutic targeting of these pro-inflammatory factors has not yielded meaningful improvements in cachexia management. More recently, the impact of immune cells in the tumour mass (tumour-associated macrophages) and host circulation (myeloid suppressor cells) has garnered much interest with regards to their role in immune tolerance in cancer. However, their role in the generation of systemic inflammation and cancer cachexia is underexplored and outstanding questions remain. This review summarises the key mediators and targets of immune dysfunction in cancer cachexia. Here we describe the host response including skeletal muscle wasting; highlight the current knowledge gap areas; and report the results of previously trialled immunotherapies. A greater understanding of complex interaction between the tumour, immune system and peripheral tissues in the genesis and maintenance of cancer cachexia is a key step in n identifying future therapeutic targets.

Keywords: Cancer cachexia, interleukins, macrophages, immunotherapy

INTRODUCTION

Cachexia is "a multifactorial syndrome defined by an ongoing loss of skeletal muscle mass (with or without loss of fat mass) that cannot be fully reversed by conventional nutritional support and leads to progressive

functional impairment"[1]. Cachexia has a negative impact on a large proportion of patients with advanced cancer with it contributing to high levels of morbidity and mortality[2]. Although there still remains some debate over the formal definition of cancer cachexia, it is characterized by unintentional weight loss, muscle wasting, anorexia and fatigue[3]. Systemic inflammation is a key driver of cancer cachexia and has been advocated as a core nutritional assessment in patients with cancer[4,5]. Pro-inflammatory cytokines are activated by the tumour mass, and act both centrally (through anorexia) and peripherally (by skeletal muscle wasting) to result in host nutritional depletion[6]. Tumour and host-derived factors thus lead to a chronic inflammatory and impaired immune state[7]. Immunosuppression is a large problem in cachectic cancer patients contributing to reduced responses to surgical and oncological outcomes[8]. The dysfunction of the immune system is complex and involves multiple mechanisms characterised by a reduction in monocyte, macrophage, dendritic and natural killer (NK) cell function, ultimately leading to susceptibility to infections, and therefore, an overall increase in morbidity[9].

In short, it has long been apparent that systemic inflammation plays a role in the pathogenesis of cancer cachexia. The successful therapeutic targeting of systemic inflammation requires a better understanding of the involved mediators and the link between tumour and immune tissues. This review aims to describe some of the key elements of immune dysfunction in cachexia and give an overview of previously trialled immunotherapies.

PRO-CACHECTIC CYTOKINES

TNF-α

TNF-α (previously known as "cachectin"[10]) was initially held responsible for causing most of the metabolic derangements and clinical features of cachexia. TNF-α is released by many types of cell, including activated macrophages, CD4+, neutrophils, mast cells, eosinophils and neurons. In particular, it can be produced by tumour, immune and stromal cells to induce growth and survival advantage in the tumour microenvironment[11]. Its expression can ultimately lead to anorexia, muscle and adipose wasting, loss of appetite, increased energy expenditure and insulin resistance in both patients with various types of cancer, and the Colon-26 carcinoma mouse model of cancer cachexia (C26)[12]. Many of the effects of TNF-α arise through activation of NFκB, which in turn leads to activation of the ubiquitin-proteasome pathway and skeletal muscle degradation[13]. It also acts to induce oxidative stress and nitric oxide species (NOS). Experimental evidence suggests TNF-α can induce adipose wasting in white adipose tissue through inhibition of lipoprotein lipase (LPL), suppression of transcription and promotion of lipolysis[14,15] as well as stimulation of thermogenesis through increased expression of UCP2 and UCP3 in skeletal muscle.

The role of TNF-α in mediating many of the effects of cancer cachexia was initially supported by evidence that intraperitoneal injection of soluble recombinant human TNF-receptor antagonist was able to improve food intake and thus lead to weight gain in tumour-bearing rats[16]. Lewis lung carcinoma (LLC) mice deficient in TNF-α receptor protein type 1 showed a reduction in muscle wasting compared with LLC wild-type mice despite similar levels of TNF-α being detected in the serum[17]. Treatment with antioxidants or NOS inhibitors was shown to increase body weight and prevent muscle wasting in mice[18]. Despite this, TNF inhibition alone in animals has not been shown to be sufficient to reduce or reverse the cachectic process indicating that, although it is involved in the pathogenesis of cancer cachexia, it is not solely responsible[19].

Studies in patients with cancer, have also not been successful. In particular adipocytes taken from cancer patients, showed no decrease in LPL messenger RNA (mRNA) or LPL enzyme activity[20]. Some studies have shown raised TNF-α levels in the serum of patients with pancreatic cancer associated with weight loss, whereas other studies in patients with terminal cancer showed no association between TNF-α and weight loss[21,22]. Others have shown that TNF-α correlates with stage of disease or tumour size rather than degree

of weight loss[23]. These discrepancies between studies may be due to differences in measuring techniques, possible auto or paracrine roles for TNF-α in adipose tissue, or heterogeneity between patients, sexes and tumours.

In summary, TNF-α is involved in systemic inflammation, but as cachexia is likely to be multifactorial, it is difficult to implicate TNF-α as the sole cause. More clinical studies are required to fully isolate its effects in patients.

Interferon gamma

Interferons are multifunctional cytokines that block viral infections and affect cell proliferation and differentiation[24]. Interferon gamma (IFNγ) is produced by activated T and NK cells, and is arguably the most potent monocyte-macrophage activating factor[25]. In the context of cancer, tumour-infiltrating lymphocytes (TILs), which have shown to be of particular importance in tumour immunosurveillance, are the main source of IFNγ[26]. There is an overwhelming body of evidence for both beneficial and detrimental roles of IFNγ in a range of diseases, including cancer. However, its role in patients with cachexia is a relatively underexplored area.

Several animal studies have indicated a central role for IFNγ in the pathogenesis of cachexia. Central administration of rat interferon resulted in decreased food intake whereas peripheral administration failed to do so[27]. Mice overexpressing IFNγ producing tumour cells developed loss of body weight, atrophy of adipose tissue, and reduced appetite, all of which were then reversed by pre-treatment of the mice with anti-IFNγ antibodies[28]. Mice with LLC also demonstrated a reduction in weight loss after treatment with anti-IFNγ antibody, significantly reducing fat wasting in particular[29]. In rats that had received transplants of MCG 101 sarcoma, anti-IFNγ antibody reduced weight loss, but the effect of treatment was short-lived[30]. Similarly to TNF-α, IFNγ has been shown to inhibit LPL activity in adipocyte cells *in vitro*, as well as that of glycerol phosphate dehydrogenase in cultures of rat adipocytes[31].

IL-1α

Levels of IL-1α have been shown to be increased in animal models of cachexia. It is thought to cause similar effects to that of TNF-α[32]. IL-1α is a pro-inflammatory cytokine produced mainly by macrophages and endothelial cells and is known for being a trigger of the acute phase response, thus playing a role in cancer pathogenesis, as well as shock and autoimmune disorders[33]. In a similar fashion to other cytokines discussed, it is also able to inhibit LPL activity and stimulate lipolysis in cultured adipocytes[34]. The ability of IL-1 to induce anorexia is thought to be due to a central effect on appetite suppression[35] involving blockade of neuropeptide Y. It also increases plasma concentrations of tryptophan and serotonin leading to early satiety and suppression of hunger[36].

Again, there is evidence for the role of IL-1α in animal models of cachexia, but little in humans. IL-1α can induce cachexia and anorexia in rats. IL-1α treated rats showed loss of body weight. Administration of IL-1α receptor antagonist (IL-1rα) to tumourbearing rats however, did not result in any improvement in body weight[37]. Following direct tumour injection with IL-1rα, C26 mice demonstrated significantly reduced weight loss (without an effect on tumour burden) compared with mice who had systemic injection[38]. Cultures of C26 cells also demonstrated raised levels of IL-6 after stimulation with IL-1α, which were suppressed by monoclonal antibody to IL-6[38]. In tumour samples from patients undergoing surgical resection for upper gastrointestinal malignancy, IL-1β and IL-6 were also significantly overexpressed in the cancer specimens, at both mRNA and protein levels, compared with control mucosa. Protein levels were seen to correlate with CRP, indicating that tumour may be the source of IL-1β[39].

IL-1β

IL-1β is a proinflammatory cytokine released by macrophages. It regulates the expression of other cytokines including IL-6 and IL-12. Recently, the loss of p53 in cancer cells from breast cancer mouse models have been shown to induce the secretion of WNT ligands that stimulate tumour associated macrophages (TAMs) to produce IL-1β, therefore helping to drive systemic inflammation. Macrophages were prevented from secreting IL-1β by pharmacological and genetic blockade of WNT secretion in p53 null cancer cells. This blockade also resulted in decreased neutrophilic inflammation and metastasis formation[40]. These findings therefore suggest an important potential future role for personalised immune therapy in patients with cancer cachexia.

IL-1β has also been better associated with the clinical features of cachexia such as anorexia, weight loss and sarcopenia than other cytokines such as IL-6 in a study of 83 advanced cancer patients[41]. Patients with gastric cancer cachexia have also been shown to have a higher prevalence of IL-1B+3954 T allele than those without indicating that patient genotype plays a role on immunological regulation of cancer cachexia[42].

IL-6

IL-6 can target adipose tissue, skeletal muscle, gut, and liver tissue, which can all affect cachectic patient body composition. It signals through the membrane bound receptor gp130 found in most tissues in the body[43]. Once bound to its receptor, it activates JAK tyrosine kinase leading to phosphorylation of tyrosines and the binding of STAT proteins. STAT proteins can translocate to the nucleus and increase the transcription of genes involved in immune function, cell proliferation, differentiation and apoptosis[43].

Several mouse cancer models have clearly demonstrated that blocking IL-6 and associated signalling can attenuate cachexia progression. Deletion of the *IL-6* gene in the APCMin/+ mouse prevented the development of cachexia[44]. IL-6 when secreted by tumour cells can also increase autophagy in myotubes when joined with soluble IL-6 receptor[45]. IL-6 trans-signalling through the soluble IL-6R has the potential to amplify IL-6 signalling in the cachectic patient and has been shown to be involved in cross-talk between tumour, muscle and adipose tissue in genetic mouse models of pancreatic cancer cachexia[46]. Autophagy and increasing IL-6 levels have been associated with poor prognosis and weight loss in lung cancer patients[45]. It has also been shown to be the key cytokine that regulates the hepatic acute phase response in patients with pancreatic cancer cachexia[47]. IL-6 remains a promising therapeutic target in cancer cachexia but a better understanding of its direct and indirect effects, as well as tissue specific actions, is required.

CYTOKINE GENOTYPE

The presence and concentration of (potentially) pro-cachectic cytokines in cancer patients appear to be dependent not only on the type of tumour, but also on the burden of disease present, and patient specific factors such as age, sex and genotype. It is still not fully understood why patients with the same histological disease may vary with regards to the presence and severity of cachexia. Genetic variation in immunity is one possible reason. Specific single nucleotide polymorphisms in the *IL-1*, *IL-6* and *IL-10* genes have been associated with cachexia in gastrointestinal cancers[48]. The 1082G allele in the IL-10 promoter was validated in an independent cohort. This was shown to be more prevalent in Myc/mTOR-driven mouse models of cachexia as well as cachectic colorectal cancer patients[48]. The C allele of the rs6136 polymorphism in the P-selectin gene has also been associated with weight loss and low CT muscularity in a large group of cancer patients[49]. These results suggest a role for the immune system in the complex presentation of cachexia.

CELLS

Myeloid derived suppressor cells

Many studies have now suggested that tumour infiltrating immune cells (those which are mainly of myeloid origin) are able to differentiate into cells which then promote tumour growth and metastasis

through their ability to induce systemic inflammation[50]. Tumours can grow through myelopoiesis and the successful evasion of tumour cells from both the innate and adaptive immune systems[51]. However, the progression of cancers appears dependent on tumour-associated myeloid cells through their ability to promote angiogenesis and tissue remodelling[52]. This apparent immunosuppression has been linked to the development of cachexia in very few studies, despite tumour-induced immunosuppression being well documented in the literature[53].

Myeloid derived suppressor cells (MDSC) may play a role in tumour-related immunosuppression. Tumour-induced amplification of the myeloid compartment leads to the expansion of myeloid-derived suppressor cells. MDSC's are immature myeloid cells in various stages of differentiation, but are not fully differentiated neutrophils, monocytes/macrophages or dendritic cells. They are found in the bone marrow, spleen, lymph nodes and tumours[50]. Their mechanisms of action are not fully understood but they are thought to be immunosuppressive and to play a role in the over production of cytokines and inflammatory mediators, which may contribute to cachexia.

MDSC expansion in 4T1 breast carcinoma-bearing mice is associated with the induction of the hepatic acute phase protein response and altered fat metabolism[53]. This response is also seen in the C26 and LLC mouse models. The pro-cachectic acute phase response is not seen, however, in 66C4 subclone of 4TI mice in which MDSC expansion does not occur[53]. Defects in myeloid cell-mediated inflammation has also been shown to result in reduced expression of pro-inflammatory cytokines in the serum of mice with hepato-cellular carcinoma[54]. Interestingly, this led to enhanced loss of adipose tissue and decreased macrophage number in visceral adipose tissue, suggesting a possible local role for macrophages in the regulation of cancer-induced fat loss[54]. These findings imply that myeloid cell-mediated inflammation confers a beneficial function in these rodents, and may provide a potential explanation for the failure of several anti-inflammatory drugs in treating cachexia. Although a direct link between the development of cachexia and MDSC's has not been proven, the above studies have suggested that the development of cancer cachexia is partly explained by the expansion of immature myeloid populations associated with the tumour.

TAMs

TAMs increase tumour progression and metastasis and suppress anti-tumour immune functions[55]. Monocytes from blood infiltrate the tumour and are primed by the tumour microenvironment to exert these effects[55]. The immune cells within the tumour's microenvironment consist of myeloid-derived suppressor cells, NK cells, dendritic cells, T cells and macrophages[56]. It is this infiltrate that contributes to tumour growth and the release of cytokines that promote the pro-cachectic environment. TAMs are recruited via cytokines and chemokines and suppress the activity of cytotoxic T-lymphocytes via programmed cell death 1 ligand 1 (PD-L1) or B7-H4 and other receptors[57]. Activated macrophages also secrete cytokines leading to the activation of several complex cascades, thereby increasing inflammatory status[58]. The chemokine monocyte chemotactic protein-1 is possibly responsible for the migration of monocytes to adipose tissue in chronic inflammation[59]. The mechanisms by which macrophages modulate adipocyte function in cachexia are still unclear.

TILs

TILs are often found in tumours and are thought to reflect an immune response against the tumour. Many studies report a survival benefit associated with the presence of TIL, suggesting they may delay tumour progression. CD3+ and CD8+ TILs in particular have been identified as having a positive effect on prognosis[60].

IMMUNE SYSTEM BIOMARKERS

The Neutrophil: Lymphocyte ratio is a prognostic indicator in cancer. Neutrophils increase the inflammatory reaction to pathogens but also interact with cancer cells to produce cytokines and effector

molecules (e.g., VEGF) that are able to stimulate angiogenesis and promote tumour growth[61]. Activated neutrophils can move from the circulation to the tumour site to release reactive oxygen species that in turn can lead to further DNA damage. They also have anti-tumour roles through antibody-mediated cytotoxicity of tumour cells[62].

C-reactive protein is an acute phase non-specific inflammatory marker that can be elevated in response to infection, surgery or malignancy. It is produced by the liver in response to increased levels of IL-6 released by activated macrophages, as well as IL-1 and TNF-α[63]. The Glasgow Prognostic Score utilises raised CRP and hypalbuminaemia to predict those patients with systemic inflammation as part of cancer cachexia and who have a poor outcome. It has been examined in more than 60,000 patients and has been shown to have independent prognostic value[64].

IMMUNOTHERAPEUTIC AGENTS FOR CANCER CACHEXIA

Immunotherapeutic agents for cancer cachexia have yielded mixed results. The different forms of immunotherapy are discussed in detail below.

TNFα inhibitors

There are currently several TNF inhibitors in use for the management of diseases such as rheumatoid arthritis (RA), psoriasis and inflammatory bowel disease, namely etanercept, infliximab and adalimumab[65]. These drugs have revolutionised the treatment of RA but have also offered insights into the role that TNF-α plays in cachexia. In RA patients, they attenuate the hepatic acute phase response and, importantly, improve patients quality of life[65,66]. They have also been shown recently to prevent worsening of the disease and restore fat free mass[67]. These drugs are now used to treat many thousands of patients and have been shown to be effective at blocking TNF-α, but are not effective in treating cancer-induced cachexia[62-64]. The feature common to all of these diseases is chronic inflammation due to exaggerated production of pro-inflammatory cytokines. Etanercept has showed some promising results in improving fatigue in a small cohort of cancer patients[68]. A phase I/II study was conducted on pancreatic cancer patients comparing etanercept and gemcitabine with gemcitabine alone. A small increase in progression free survival was seen associated with higher plasma IL-10 levels, but there was no significant improvement in 6 month progression-free survival compared with gemcitabine alone[68]. A placebo-controlled double-blind trial was also undertaken in 63 patients with incurable malignancy and weight loss of > 2.27 kg over 2 months or daily intake of < 20 calories/kg body weight. Weight gain was found to be minimal in both arms with comparable survival times. Treatment was associated with higher neurotoxicity[69]. In this trial, therefore, etanercept was not effective in the treatment of cachexia in patients with advanced disease.

Infliximab has been used in a phase II placebo controlled randomised study in patients with stage II-IV pancreatic cancer[70]. Patients were given either 3 mg/kg or 5 mg/kg infliximab with gemcitabine or placebo with gemcitabine. The mean change in lean body mass was + 0.4 kg for those on placebo, + 0.3 kg for those receiving 3 mg/kg of infliximab, and + 1.7 kg for those receiving 5 mg/kg of infliximab. No statistically significant differences were seen, however[70].

Another agent with anti-inflammatory activity is OHR/AVR118, a broad-spectrum peptide-nucleic acid immune modulator that targets both TNF-α and IL-6[71]. A phase II study involving patients with advanced cancer and cachexia showed an improvement in anorexia, dyspepsia, strength, and depression[71].

Anti-IFNγ treatments

Anti IFNγ treatments have been effective in reverting cachexia in the LLC mouse model[29]. There have been no trials undertaken in cancer patients, mainly due to the fact that this type of therapy requires a dose to completely block the action of IFNγ, and at present, such a treatment programme would be very expensive. The only trial conducted in patients with cachexia due to sepsis showed no benefit[72].

IL-1/6 inhibitors

The IL-1 pathway has been previously targeted in humans with the recombinant human IL-1 receptor antagonist Anakinra and the neutralising monoclonal anti-IL-1 antibody Canakinumab. Anakinra, as previously discussed, has had success in rheumatoid patients but has yet to be trialled in patients with cancer[65]. However, a more specific IL-1a human monoclonal antibody, MABp1, has shown promising results in cancer. An initial dose escalation and expansion study was designed using MABp1[73]. The first dose escalation study was performed in patients with refractory cancer to assess its safety and tolerability. It identified an optimal intravenous dose which was then used in the following phase II study of forty-two patients[73]. Median plasma IL-6 concentrations decreased from baseline to week 8 ($P = 0.08$). Of those 30 patients who had an assessment of body composition, lean mass increased significantly by 1.02 ± 2.24 kg ($P = 0.02$)[73]. It was then compared to megestrol acetate in patients with advanced colorectal cancer and > 5% weight loss. Those in the MABp1 treatment arm showed a trend towards increased survival[74]. A placebo-controlled, double blind phase III study in 333 patients with advanced colorectal cancer was then undertaken which resulted in increased lean body mass as well as symptom relief (pain, anorexia, fatigue)[75].

IP1510 is a synthetic peptide IL-1 receptor antagonist. Pre-clinical studies found it to have low toxicity, and to be a potential effective treatment for cachexia. It was then trialled in advanced gynaecological cancer patients where it was well tolerated, and it significantly improved patient anorexia, depression and physical performance. Weight stabilisation or gain was seen in 17 of the 26 enrolled patients[76]. Interpretation of the current data is limited because the study was neither randomised nor controlled. However, further larger trials are to be initiated targeting IL1 in cancer cachexia[74].

Studies involving IL-6 antibodies have been undertaken in patients with advanced non-small cell lung cancer. The humanised monoclonal IL-6 antibody Clazakizumab [ALD518] has shown beneficial results in increasing haemoglobin levels and preventing loss of lean body mass. Fatigue scores were also improved compared with controls[77]. There are, however, no phase III trials underway.

Immunotherapeutic agents continue to be a promising treatment for cancer cachexia and may be added to the multi-modal management approach for this complex syndrome.

Standard cancer immunotherapy

Anti-cancer immunotherapy including bermekimab above has been shown to improve outcomes in a range of tumour types, including melanoma, lung and bladder cancers, many of which respond poorly to traditional agents. Some of these therapies are, however, poorly understood. Patients with cancer cachexia have been shown to respond poorly to some immunotherapies such as immune check point inhibitor therapy, likely due to elevated clearance or the establishment of primary resistance[78]. In two large clinical trials involving patients with melanoma and non-small cell lung cancer, there was a paradoxical association between plasma clearance of Pembrolizumab [a programmed cell death protein inhibitor (PD-1)] and poor overall survival. Those patients who responded poorly were noted to have reduced body weight and low albumin, suggesting that the presence of cachexia rendered these patients unable to respond to Pembrolizumab[76]. The hypoalbumaemia was hypothesised to explain the elevated plasma clearance, and therefore the dose was increased in these patients to counteract this apparent resistance, but it did not result in improved outcomes. Studies in lung cancer patients treated with immunotherapy have shown that decreases in pre-treatment BMI, weight loss and high neutrophil:lymphocyte ratio were associated with significantly shorter progression-free survival[79]. However, other studies in lung cancer patients have also shown that two thirds of those receiving PD-1 and PD-L1 immune check point inhibitors experienced stability or an increase in their skeletal muscle index[60]. Thus, although at first glance immunotherapy would seem to be a natural antidote to cancer cachexia, it is difficult to unravel the clinical impact of immunotherapy from the known adverse outcomes of any cancer patients with poor nutritional status.

These studies therefore raise the possibility that immunotherapies could represent effective anti-cachexia agents but that synchronous multimodal and nutritional anti-cancer treatments may be required to establish or enhance their overall effectiveness.

A key consideration in using immunotherapy may be the inflammatory status of the host. It has been demonstrated that the host inflammatory status influences the efficacy of therapy with inflamed patients most likely to benefit from therapies with an anti-inflammatory mode of action[61]. Similar to the call to "stage the tumor, stage the host"[62] it is now key that treatment stratification is based on the inflammatory status of the patient and this is now being used as a mandatory measure in clinical trials in some tumour groups[63]. It is clear that whilst immunotherapies as a treatment for cancer cachexia, there is a necessity to ensure patients who receive these are those who are most likely to benefit.

CONCLUSION

The pathogenesis of cancer cachexia is highly dependent on the patient's immune response. The interplay between inflammatory cytokines (such as TNF-α, IFNγ and interleukins) and pro-cachectic factors contributes to the complex aetiology. These cytokines are produced by the host in response to the tumour, as well as by the tumour itself. Many treatments have tried to regulate the immune response in cachexia but have largely been unsuccessful, perhaps in part due to the multifactorial nature of cachexia, and the observed heterogeneity of patient factors. Large-scale clinical studies are needed to prove whether neutralisation of deleterious cytokines or direct receptor antagonism in combinatorial treatment regimens is an effective therapeutic approach to improve patient outcomes or to reverse muscle loss in cancer cachexia.

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Authors' contributions

Drafted the manuscript: Miller J
Critically revised the manuscript and gave final approval for the version to be published: Laird BJA, Skipworth RJE

Availability of data and materials

Not applicable.

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The authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

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Validated screening tools for the assessment of cachexia, sarcopenia, and malnutrition: a systematic review

Janice Miller,[1] Liz Wells,[2] Ugochinyere Nwulu,[3] David Currow,[3,4] Miriam J Johnson,[3] and Richard JE Skipworth[1]

[1]Clinical Surgery, University of Edinburgh, Royal Infirmary of Edinburgh, Edinburgh, United Kingdom; [2]Diabetes, Endocrinology, and Metabolism, Hull Royal Infirmary, Hull, United Kingdom; [3]Wolfson Palliative Care Research Center, Hull York Medical School, University of Hull, Hull, United Kingdom; and [4]Improving Palliative Care through Clinical Trials (IMPACCT), Faculty of Health, University of Technology, Sydney, New South Wales, Australia

ABSTRACT

Background: There is great overlap between the presentation of cachexia, sarcopenia, and malnutrition. Distinguishing between these conditions would allow for better targeted treatment for patients.

Objectives: The aim was to systematically review validated screening tools for cachexia, sarcopenia, and malnutrition in adults and, if a combined tool is absent, make suggestions for the generation of a novel screening tool.

Design: A systematic search was performed in Ovid Medline, EMBASE, CINAHL, and Web of Science. Two reviewers performed data extraction independently. Each tool was judged for validity against a reference method. Psychometric evaluation was performed as was appraisal of the tools' ability to assess the patient against consensus definitions.

Results: Thirty-eight studies described 22 validated screening tools. The Cachexia score (CASCO) was the only validated screening tool for cachexia and performed well against the consensus definition. Two tools assessed sarcopenia [the Short Portable Sarcopenia Measure (SPSM) and the SARC-F (Strength, Assistance with walking, Rise from a chair, Climb stairs, and Falls)] and scored well against the 1998 Baumgartner definition. The SPSM required large amounts of equipment, and the SARC-F had a low sensitivity. Nineteen tools screened for malnutrition. The 3-Minute Nutrition Score performed best, meeting consensus definition criteria (European Society for Clinical Nutrition and Metabolism) and having a sensitivity and specificity of >80%. No tool contained all of the currently accepted components to screen for all 3 conditions. Only 3 tools were validated against cross-sectional imaging, a clinical tool that is gaining wider interest in body-composition analysis.

Conclusions: No single validated screening tool can be implemented for the simultaneous assessment of cachexia, sarcopenia, and malnutrition. The development of a tool that encompasses consensus definition criteria and directs clinicians toward the underlying diagnosis would be optimal to target treatment and improve outcomes. We propose that tool should incorporate a stepwise assessment of nutritional status, oral intake, disease status, age, muscle mass and function, and metabolic derangement. *Am J Clin Nutr* 2018;108:1196–1208.

Keywords: cachexia, sarcopenia, malnutrition, screening, assessment

INTRODUCTION

Unintentional weight loss (UWL) as a form of nutritional depletion is commonly seen in aging, cancer, and many chronic diseases. The main subtypes can be categorized into 3 primary syndromes: cachexia, age-related sarcopenia, and malnutrition. However, it is not clear whether existing screening tools are able to distinguish between these 3 conditions. This is due in part to the complex overlap between them. Loss of muscle mass is a key feature in both cachexia and sarcopenia, but patients with sarcopenia

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MJJ and RJES contributed equally to this work as final authors.

Address correspondence to JM (e-mail: j.miller@ed.ac.uk).

Abbreviations used: CASCO, Cachexia score; CT, computed tomography; DXA, dual-energy X-ray absorptiometry; MNA, Mini Nutritional Assessment; SARC-F, Strength, Assistance with walking, Rise from a chair, Climb stairs, and Falls; SGA, Subjective Global Assessment; SPSM, Short Portable Sarcopenia Measure; UWL, unintentional weight loss; 3-MinNS, 3-Minute Nutrition Score.

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are not necessarily cachectic. Sarcopenia can occur simply with aging and leads to functional decline (1, 2). Cachexia involves complex metabolic pathways involving systemic inflammation and muscle and fat wasting and must be present in association with a chronic disease (3). Cachexia differs from malnutrition in that it cannot be reversed by simple nutritional support (4). There are many definitions for each condition, with nutritional depletion playing a part in each, therefore making it difficult to separate them out (1–4). These conditions are also often not noticed in their earlier phases but do become apparent after a critical event or development of disability (5).

More than 70 nutritional screening tools for use in hospitals have been developed to facilitate easy screening or assessment of a patient's nutritional status or to predict poor clinical outcome related to UWL. Despite increasing research, there appears to be a lack of a practical and implementable clinical screening tool to support diagnosis (6). In the general community, the European Society for Clinical Nutrition and Metabolism endorses the use of the Malnutrition Screening Tool (MUST) (6, 7) and the Nutritional Risk Screening (NRS-2002) (8) and the Mini Nutritional Assessment (MNA)–Short Form for the elderly (9, 10). Some tools claim to have been developed to screen specific target groups; however, there are currently no disease-specific recommendations. There is no international consensus on a single "best tool" to identify all 3 syndromes across populations. The use of different tools in different studies makes drawing any conclusions about their comparison and meta-analyses difficult.

Current diagnostic methods for sarcopenia and cachexia include the assessment of body anthropometric measures using either BMI or estimated weight loss, or by direct assessment of muscle and fat mass using dual-energy X-ray absorptiometry (DXA), bioelectrical impedance analysis, computed tomography (CT), or MRI scanning. Although the latter 2 radiographic modalities are accurate, they are impractical and expensive and some expose the patient to radiation. This diagnostic approach to detect the presence of sarcopenia is time consuming, expensive, and requires highly specialized equipment (11). Therefore, a screening tool that is implementable in a larger population that allows for early detection is important. This approach would highlight the potential for further assessment with early biomarkers, thus allowing prophylactic intervention in malnutrition and driving further research in sarcopenia and cachexia.

We aimed to systematically review validated screening tools for the general adult population to enable clinicians to distinguish between the 3 syndromes. The specific strengths and limitations of each tool were assessed, as was the appropriateness of the validation population. Through psychometric evaluation and assessment of the tools against the agreed-upon consensus definitions, we also investigated if any one single tool could be used for the simultaneous assessment of all 3 syndromes.

METHODS

Methods for conducting systematic reviews of the effectiveness of interventions have been well described. In accordance with PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines (12), we applied the principles to systematically reviewing validated screening tools used in the assessment of cachexia, sarcopenia, and malnutrition.

Literature review

A systematic search was performed on 7 August 2018 in Ovid Medline (1946–2017; https://ovidsp.ovid.com), EMBASE (1974–2017; https://www.embase.com), CINAHL (Cumulative Index to Nursing and Allied Health Literature; https://www.ebscohost.com/nursing/products/cinahl-databases/cinahl-complete), and Web of Science (wok.mimas.ac.uk). Relevant articles were identified by title and abstract. Reference lists of review articles were also hand-searched. Double data extraction was performed by 2 reviewers independently to ensure consistency. Any disagreements were settled by a third reviewer.

The basic search strategy was "Sarcopenia" OR "Cachexia" OR "Malnutrition" AND "screening" AND "validation study" using MeSH (Medical Subject Heading) terms and keywords appropriate to each database. No language restriction was imposed. The search was designed to be broad to ensure all validated tools were identified. A full copy of the search used for Medline can be found in the **Supplemental Material 1**. There were no disease-specific limits.

Inclusion and exclusion criteria

Studies were included if they had developed a screening tool that had been validated for the screening of either cachexia, sarcopenia, or malnutrition in adults (**Table 1**). Disease-specific tools were included. Studies were excluded if the tools had not been validated or if they assessed malnutrition in children or obesity in adults. Studies that described modified versions of pre-existing tools were also excluded because this was out of the scope of this review. It was intended that studies that included <25 patients should be excluded because they were unlikely to yield robust, generalizable psychometric results; however, no studies with numbers smaller than this were found.

Assessment of validity

Studies required evaluation of ≥2 of the following psychometric characteristics: content validity, construct validity (e.g., including convergent validity, discriminant validity), test-retest reliability, internal consistency, responsiveness, factor analysis, or criterion validity. Primary criteria used to evaluate the tools were construct validity and responsiveness.

Criterion and construct validity, reference method

Studying the validity of a tool usually involves comparison to a gold standard. Although many research groups are now using cross-sectional imaging to investigate UWL, there is currently no perfect gold standard. Studies used different reference methods to validate their tools (e.g., DXA and assessment by a health professional). The tools Subjective Global Assessment (SGA) and the MNA are the tools currently recognized as the industry standard and were therefore considered valid references (4, 6). The term "criterion validity" was used for these comparisons.

Less-valid reference methods, including the use of other screening tools and blood tests (e.g., albumin) which can be influenced by other factors including inflammation and acute disease, were included because many research groups vary in their opinion on the optimal reference method (13). These comparisons were termed "construct validity."

TABLE 1
Inclusion criteria

Criteria	Description
Types of participants: adults (aged >18 y) undergoing routine screening for cachexia, sarcopenia, or malnutrition	Includes patients with advanced cancer and end-stage cardiac, renal, and liver disease
Types of tools: validated, quantitative measurements of cachexia, sarcopenia or malnutrition	Tools developed for clinical or research purposes; completed by health care professionals
Psychometric evaluation (demonstration of ≥2 criteria)	
Content validity	Breadth of scope of tool: to what extent does it appear to capture the relevant aspects of unintentional weight loss; are there gaps?
Construct validity, including convergent validity, discriminant validity	How well the tool relates to other measures of the same construct; lack of correlation with dissimilar or unrelated constructs or variables
Test-retest reliability	How consistent an individual's scores are over a defined time period presuming weight stays constant
Internal consistency	How closely related are the different items in the tool?
Responsiveness	Ability to detect clinically meaningful change for individuals
Factor analysis	For a tool comprising several items, a way of grouping them into factors which may tap into a particular construct
Criterion validity	A shortened version of a scale, concurrent validity with the longer version that has been validated

Predictive validity

Predictive validity was assessed as the ability of the tool to predict the probability of a better or worse clinical outcome due to nutritional risk.

Diagnostic criteria

Tools were also assessed for their ability to identify the risk of cachexia, sarcopenia, or malnutrition by comparison of their components against the components of each set of chosen diagnostic criteria (**Table 2**).

Assessment of bias

Assessment of bias was made with the use of a form of the Newcastle-Ottawa scale adapted for cross-sectional studies (14). Each study was scored out of 10 possible points, and a study with a score of <5 was considered to be at high risk of bias. Full details of the scoring used can be found in the **Supplemental Material 2**.

Secondary criteria

Secondary criteria included face validity, development and content validity, factor analysis, test-retest reliability, internal consistency, and respondent and administrative burden (the time and effort required to complete the tool). These are also summarized in Table 1. Data were extracted on the study participants, the tool used, and psychometric evaluations (inclusion criteria, Table 1). An assessment of sensitivity and specificity was made. A value >80% was considered good, 60–80% fair, and <60% poor. Agreement was also assessed as follows: 0.9–1.0 = excellent, 0.80–0.89 = good, 0.61–0.79 = fair, and <0.60 = poor.

RESULTS

Principal findings

Thirty-eight studies were included that described the validation of 22 screening tools. The majority of studies were excluded because they described nonvalidated tools. This is summarized in **Figure 1**.

The Cachexia score (CASCO) was the only screening tool for cachexia that had been validated. It performed well against diagnostic criteria (3), but sensitivities and specificities were not recorded. Only 2 tools assessed sarcopenia [the Short Portable Sarcopenia Measure (SPSM) and the SARC-F (Strength, Assistance with walking, Rise from a chair, Climb stairs, and Falls)] and scored well against the agreed definition (1). However, the

TABLE 2
Summary of proposed diagnostic criteria for identification of cachexia, sarcopenia, and malnutrition[1]

Syndrome	Diagnostic criteria
Cachexia	Weight loss >5% or weight loss >2% in individuals already showing depletion according to current body weight and height [BMI (in kg/m^2) <20] or skeletal muscle mass (sarcopenia)[2]
Sarcopenia	Loss of function: 6-min walk <400 m or gait speed <1.0 m/s Muscle mass: low appendicular lean mass or height[3] (2 SDs below the mean diagnostic on DXA[4])
Malnutrition	Protein-energy deficiency: risk indicated by low BMI <18.5 or weight loss >10% (indefinite time)/5% over last 3 mo and BMI <20 (if aged <70 y)/<22 (if aged >70 y) or FFMI <15 and 17 kg/m^2 in men and women, respectively[5]

[1]DXA, dual-energy X-ray absorptiometry; FFMI, fat-free mass index.
[2]Data from reference 3.
[3]Data from reference 2.
[4]Data from references 1 and 2.
[5]Data from reference 4.

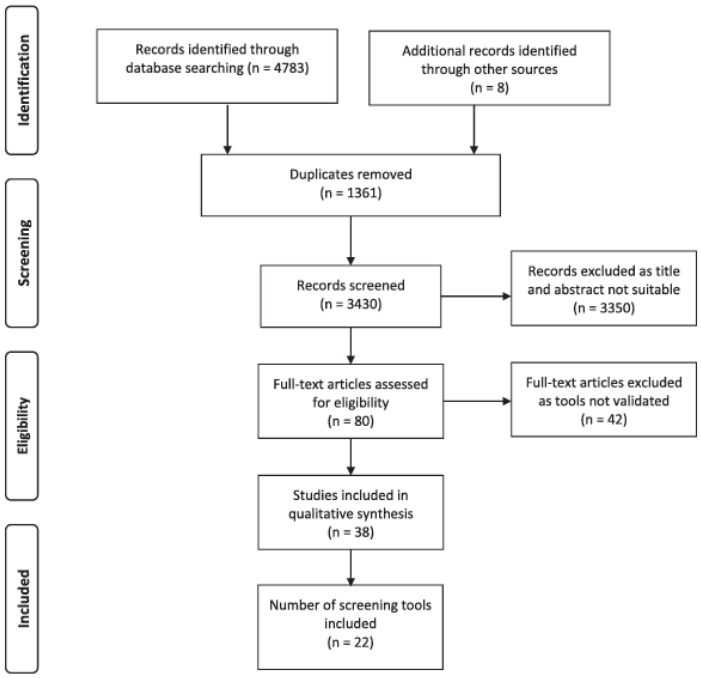

FIGURE 1 PRISMA flow diagram (12). PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses.

SPSM required the transport of equipment and the SARC-F had very low sensitivity. Both were validated for use in the outpatient setting. Nineteen tools screened for malnutrition. The 3-Minute Nutrition Score (3-MinNS) proved to be the best, scoring well against the consensus definition (European Society for Clinical Nutrition and Metabolism) as well as having sensitivities and specificities >80%. There was no single validated tool that adequately screened for all 3 conditions. A critical appraisal of all tools can be found in **Table 3**.

Tools with evidence of validity, reliability, and acceptability

The available validity, reliability, and acceptability data are summarized in **Tables 4** and **5**. **Table 6** assesses how well each tool encompasses the criteria in the chosen definitions. Assessment of bias is shown in **Table 7**.

Sarcopenia

In total, 2 tools were found that were validated for the assessment of sarcopenia (SPSM and SARC-F). Three other tools assessed muscle function, but no other tools made an assessment of muscle strength, mass, or wasting. Both tools that were validated for the assessment of sarcopenia were done so in the community setting. They agreed with the Society on Sarcopenia, Cachexia and Wasting Disorders diagnostic criteria, but the SARC-F showed variation in agreement against the 3 consensus definitions it was validated against (European Working Group on Sarcopenia in Older People, International Working Group on Sarcopenia criteria, and the Asian working group for sarcopenia). The SARC-F had good specificity (94.2–99.1%) but poor sensitivity (3.8–9.9%, dependent on sex) and also showed good agreement (0.78–0.90). Values for the SPSM were not assessed.

Cachexia

Only 1 tool had been validated for the screening of cachexia—the CASCO. Overall, 6 tools quantified weight loss within a specified time frame, with a further 3 quantifying it within an unspecified time frame. Sixteen tools characterized weight loss as unintentional. Only 7 tools asked about the presence of underlying disease, and only the CASCO took into account the presence of elevated inflammatory markers and quality of life. Sensitivities and specificities were not recorded for the CASCO, but it scored well in the assessment of its validity, with it being able to quantitatively classify stages of cachexia. Its ability to predict patient outcome was not assessed.

TABLE 3
Critical appraisal of tools to measure unintentional weight loss[1]

Study, year (ref)	Tool	Description	Validation population	Validation reference	Strengths	Limitations
Sarcopenia						
Woo et al., 2014 (15)	SARC-F	A questionnaire regarding ability to carry a heavy load, walking, rising from a chair, climbing stairs, and frequency of falls	Community-dwelling Chinese ($n = 4000$)	3 consensus definitions of sarcopenia	Not dependent on cutoff values	No assessment of muscle mass, not validated in hospital populations
Miller et al., 2009 (16)	SPSM	Portable measure that combines estimates of muscle quantity and function into a single scale	Community-dwelling African Americans ($n = 998$)	DXA	Portable	Time-consuming, equipment-dependent, muscle mass not measured
Cachexia						
Argiles et al., 2017 (17)	CASCO	Score to classify cachectic patients into 3 different groups; includes 5 components: body weight loss and composition, inflammation/metabolic disturbances/immunosuppression, physical performance, anorexia, and quality of life	Cancer patients ($n = 186$)	Assessment by oncologist	Encompasses all diagnostic criteria	Involves many questions and measurements, does not include questions on disease state
Malnutrition						
Weekes et al., 2004 (18)	BAPEN	Tool based on 4 nutritional parameters (weight, height, recent UWL, and appetite)	Acute medical and elderly care wards ($n = 100$)	Dietitian	Quick and easy	Percentage of weight loss not quantified
Mimiran et al., 2011 (19)	BNST	Score based on UWL, unintentional eating loss, and being unable to eat for >5 d	Medical and surgical ($n = 446$)	Dietitian	Easily completed by nursing staff	Low importance given to amount of weight loss
Laporte et al., 2015 (20)	CNST	Tool containing 2 items: weight loss and decreased food intake	Medical and surgical ($n = 150$)	SGA	Very brief, can be completed by nontrained rater	Assessed on admission only; validity of rescreening unknown
Ignacio et al., 2005 (21)	CONUT	Evaluates nutrition using albumin, cholesterol, and lymphocyte count; automated system	Medical and surgical inpatients ($n = 53$)	SGA	Simple, automated	Markers vary depending on disease state, only done in patients who have blood samples taken
Guerra et al., 2017 (22)	EDC	Screening tool based on ESPEN criteria for diagnosis malnutrition	Medical and surgical inpatients ($n = 632$)	PG-SGA	Includes FFM assessment	Very low sensitivity
Abd-El-Gawad et al., 2014 (23)	GNRI	Modified nutritional risk index for geriatric patients (based on albumin, current and previous weight)	Acute geriatrics ward ($n = 131$)	MNA	Good prognosticator, does not require capacity	Diseases associated with high mortality or hypoalbuminemia excluded
Tammam et al., 2009 (24)	INSYST	Two-tiered tool: first is a simple prescreen aiming to establish if malnourished; second provides a more detailed evaluation	Medical, surgical and oncological inpatients ($n = 61$)	MUST and MNA	Does not require height and BMI, quick and easy	Ease of completing dependent on patient's cognitive state
Ferguson et al., 1999 (25)	MST	Two questions regarding appetite and UWL	Medical and surgical inpatients ($n = 408$)	SGA	Very quick, does not require calculations	Nonspecific
Isenring et al., 2006 (26)	MST	—	Oncology outpatients ($n = 51$)	PG-SGA	—	—
Neelemaat et al., 2011 (27)	MST	—	Acute hospitalized ($n = 193$)	Malnutrition definition	—	—
Nursal et al., 2005 (28)	MST	—	Medical and surgical inpatients ($n = 2211$)	SGA	—	—
Young et al., 2013 (29)	MST	—	Elderly medical inpatients ($n = 134$)	PG-SGA	—	—
Wu et al., 2012 (30)	MST	—	Elderly inpatients ($n = 157$)	SGA	—	—
Bhuachalla et al., 2018 (31)	MST	—	Oncology patients ($n = 725$)	CT	—	—
Leiopold et al., 2018 (32)	MST	—	Rehabilitation patients ($n = 160$)	SGA	—	—
Kim et al., 2011 (33)	MSTC	Tool based on intake change, weight loss, ECOG performance status, and BMI	Oncology inpatients ($n = 1057$)	PG-SGA	Cancer-specific	Designed to be performed by dietitians, not nurses
Boleo-Tome et al., 2012 (34)	MUST	Five-step tool including BMI, unplanned weight loss, and presence of acute disease	Oncology inpatients ($n = 450$)	PG-SGA	Quick, easy	Does not pick up patients with normal BMI who are malnourished, UWL reported by patients is subjective
Leistra et al., 2013 (35)	MUST	—	Medical and surgical outpatients ($n = 2236$)	Malnutrition definition and BMI	—	—
Sharma et al., 2017 (36)	MUST	—	Acute medical inpatients ($n = 132$)	PG-SGA	—	—
Neelemaat et al., 2011 (27)	MUST	—	Elderly inpatients ($n = 198$)	Malnutrition definition	—	—
Kyle et al., 2006 (37)	MUST	—	Medical and surgical ($n = 995$)	SGA	—	—

(*Continued*)

TABLE 3
(*Continued*)

Study, year (ref)	Tool	Description	Validation population	Validation reference	Strengths	Limitations
Young et al., 2013 (29)	MUST	—	Medical inpatients ($n = 134$)	SGA and MNA	—	—
Almeida et al., 2012 (38)	MUST	—	Surgical inpatients ($n = 300$)	SGA	—	—
Velasco et al., 2011 (39)	MUST	—	Medical and surgical ($n = 400$)	SGA, NRS-2002, and MNA	—	—
Bhuachalla et al., 2018 (31)	MUST	—	Oncology patients ($n = 725$)	CT	—	—
Prasad et al., 2012 (40)	NRI	Derived from serum albumin concentration and ratio of usual to present weight	Peritoneal dialysis patients ($n = 283$)	SGA	Assesses dialysis patients at risk	Relies on previous weight; limited use with changes in fluid status
Faramarzi et al., 2013 (41)	NRI	—	Colorectal cancer ($n = 52$)	PG-SGA	—	—
Bhuachalla et al., 2018 (31)	NRI	—	Oncology patients ($n = 725$)	CT	—	—
Neelemaat et al., 2011 (27)	NRS-2002	Tool containing nutritional components of the MUST along with disease severity	Elderly inpatients ($n = 198$)	Definition of malnutrition	Includes disease severity; therefore, applicable in ITU	Ease of completing dependent on patient's cognitive state
Kyle et al., 2006 (37)	NRS-2002	—	Medical and surgical ($n = 995$)	SGA	—	—
Young et al., 2013 (29)	NRS-2002	—	Elderly medical patients ($n = 134$)	SGA and MNA	—	—
Almeida et al., 2012 (38)	NRS-2002	—	Surgical inpatients ($n = 300$)	SGA	—	—
Bauer et al., 2005 (42)	NRS-2002	—	Acute geriatrics ward ($n = 121$)	SGA and MNA	—	—
Velasco et al., 2011 (39)	NRS-2002	—	Medical and surgical ($n = 400$)	MUST, SGA, and MNA	—	—
Soderhamn et al., 2002 (43)	NUFFE	Three-point ordinal scale with 15 items assessing weight loss, dietary history, appetite, and general activity	Elderly care rehab ward ($n = 114$)	MNA	Simple because lacks anthropometric measurements	Many confounding factors in questionnaire
Duerksen et al., 2000 (44)	SGA	Assessment of nutritional status based on history and examination	Acute elderly care and elderly rehab ($n = 95$)	Geriatric and internal medicine resident, anthropometric data	Current gold standard	Reproducibility less than in nonelderly, unable to predict severe malnutrition in ESRD, requires experienced operator to carry out
Cooper et al., 2002 (45)	SGA	—	ESRD ($n = 76$)	Total-body nitrogen	—	—
Moriana et al., 2014 (46)	SGA	—	Medical and surgical inpatients ($n = 197$)	Anthropometric and biochemical data	—	—
Kruizenga et al., 2005 (47)	SNAQ	26 questions related to eating and drinking difficulties, defecation, condition, and pain	Medical, surgical and oncological inpatients ($n = 291$)	Malnutrition criteria	Corresponds to ESPEN criteria	High NPV, no outcome data
Leistra et al., 2013 (35)	SNAQ	—	Medical and surgical outpatients ($n = 2236$)	MUST	—	—
Harada et al., 2017 (48)	SNAQ	—	Oncology outpatients undergoing chemotherapy ($n = 300$)	CONUT	—	—
Neelemaat et al., 2011 (27)	SNAQ	—	Medical and surgical inpatients ($n = 2211$)	MST, MNA-SF, MUST, NRS-2002	—	—
Young et al., 2013 (29)	SNAQ	—	Elderly medical inpatients ($n = 134$)	SGA and MNA	—	—
Susetyowati et al., 2014 (49)	SNST	Six questions including weight loss, appetite, and health status	Medical and surgical inpatients ($n = 495$)	SGA	Can be done by nontrained staff	No anthropometric assessment, all subjective
Wong et al., 2011 (50)	Spinal NST	Tool that assesses 8 criteria including appetite, weight loss, and level of spinal cord injury	Spinal cord injury patients ($n = 150$)	Dietetic assessment	Disease specific	Requires specialized scales to measure paralyzed patients
Xia et al., 2016 (51)	R-NST	Nine questions assessing malnutrition risk/symptoms combined with albumin, CRP, and urea	Renal inpatients ($n = 122$)	SGA	Renal specific	Patients picked up for conditions other than malnutrition (e.g., hyperkalemia)
Lim et al., 2009 (52)	3-MinNS	Questionnaire based on diagnostic criteria for malnutrition and muscle wastage	Medical and surgical inpatients ($n = 818$)	SGA	Quick and easy	Dependent on cognitive state

[1] BAPEN, British Association for Parenteral and Enteral Nutrition; BNST, British Nutrition Screening Tool; CASCO, Cachexia score; CNST, Canadian Nutrition Screening Tool; CONUT, Controlling Nutritional Status; CRP, C-reactive protein; CT, computed tomography; DXA, dual-energy X-ray absorptiometry; ECOG, Eastern Cooperative Oncology Group; EDC, ESPEN Diagnostic Criteria for Malnutrition; ESPEN, European Society for Clinical Nutrition and Metabolism; ESRD, end-stage renal disease; FFM, fat-free mass; GNRI, Geriatric Nutrition Risk Index; INSYST, Imperial Nutritional Screening System; ITU, intensive therapy unit; MNA, Mini Nutritional Assessment; MNA-SF, Mini Nutritional Assessment Short Form; MST, Malnutrition Screening Tool; MSTC, Malnutrition Screening Tool for Cancer; MUST, Malnutrition Universal Screening Tool; NPV, negative predictive value; NRI, Nutritional Risk Index; NRS-2002, Nutritional Risk Screening; NUFFE, Nutritional Form for the Elderly; PG-SGA, Patient-Generated Subjective Global Assessment; ref, reference; R-NST, Renal Nutritional Screening Tool; SARC-F, Strength, Assistance with walking, Rise from a chair, Climb stairs, and Falls; SGA, Subjective Global Assessment; SNAQ, Short Nutritional Assessment Questionnaire; SNST, Simple Nutritional Screening Tool; Spinal NST, Spinal Nutritional Screening Tool; SPSM, Short Portable Sarcopenia Measure; UWL, unintentional weight loss; 3-MinNS, 3-Minute Nutrition Screening.

TABLE 4
Psychometric evaluation of tools to measure unintentional weight loss[1]

Scale	Environment	Context (outpatients or inpatients)	Face validity	Content validity	Factor analysis	Construct validity	Discriminant validity	Predictive validity	Test-retest	Internal consis-tency	Responsiveness	Acceptability	Time to complete
Sarcopenia													
SARC-F	Community dwelling	Outpatients	♦	–	–	♦	–	♦	–	♦	–	♦	–
SPSM	Community dwelling	Outpatients	–	–	♦	X	♦	♦	♦	♦	♦	–	♦
Cachexia													
CASCO	Oncology	Outpatients	♦	♦	♦	♦	♦	–	–	♦	♦	–	–
Malnutrition													
BAPEN	Acute medical and elderly care	Inpatients	♦	–	–	♦	–	–	♦	–	♦	♦	♦
BNST	Spinal cord injuries	Inpatients	♦	–	♦	–	–	–	♦	–	–	–	–
CNST	Medical and surgical	Inpatients	♦	–	–	♦	–	♦	♦	–	–	–	–
CONUT	Medical and surgical	Inpatients	♦	–	X	♦	–	–	–	–	♦	♦	–
EDC	Medical and surgical	Inpatients	♦	–	–	–	–	♦	–	♦	–	–	–
GNRI	Acute geriatrics	Outpatients	♦	–	♦	–	–	♦	–	–	–	♦	–
INSYST	Medical, surgical, and oncology	Inpatients	♦	♦	–	♦	–	–	♦	–	♦	♦	♦
MST	Medical, surgical, and oncology	Inpatients, outpatients	♦	♦	♦	♦	–	♦	♦	♦	–	♦	–
MSTC	Oncology	Inpatients	♦	X	♦	♦	–	–	–	–	–	X	♦
MUST	Medical, surgical, and oncology	Inpatients, outpatients	♦	♦	X	♦	–	♦	–	–	♦	♦	♦
NRI	Peritoneal dialysis and colorectal cancer	Inpatients	–	–	♦	–	–	♦	–	–	–	♦	–
NRS-2002	Elderly, medical, and surgical	Inpatients	♦	♦	–	♦	–	♦	♦	–	♦	♦	♦
NUFFE	Elderly care rehab	Inpatients, outpatients	♦	♦	♦	♦	–	♦	♦	♦	–	–	–
R-NST	Renal	Inpatients	♦	♦	♦	♦	–	–	–	–	–	X	♦
SGA	Elderly, renal, medical, and surgical	Inpatients	♦	♦	♦	♦	–	♦	♦	♦	–	–	–
SNAQ	Medical, surgical, and oncology	Inpatients, outpatients	♦	♦	–	♦	♦	♦	♦	–	♦	♦	♦
SNST	Medical and surgical	Inpatients	♦	–	♦	–	♦	♦	♦	♦	♦	♦	♦
Spinal NST	Spinal cord injuries	Inpatients	♦	–	♦	–	–	–	♦	–	–	♦	♦
3-MinNS	Medical and surgical	Inpatients	♦	♦	–	♦	♦	♦	♦	–	♦	♦	♦

[1] BAPEN, British Association for Parenteral and Enteral Nutrition; BNST, British Nutrition Screening Tool; CASCO, Cachexia score; CNST, Canadian Nutrition Screening Tool; CONUT, Controlling Nutritional Status; EDC, European Society for Clinical Nutrition and Metabolism Diagnostic Criteria for Malnutrition; GNRI, Geriatric Nutrition Risk Index; INSYST, Imperial Nutritional Screening System; MST, Malnutrition Screening Tool; MSTC, Malnutrition Screening Tool for Cancer; MUST, Malnutrition Universal Screening Tool; NRI, Nutritional Risk Index; NRS-2002, Nutritional Risk Screening; NUFFE, Nutritional Form for the Elderly; R-NST, Renal Nutritional Screening Tool; SARC-F, Strength, Assistance with walking, Rise from a chair, Climb stairs, and Falls; SGA, Subjective Global Assessment; SNAQ, Short Nutritional Assessment Questionnaire; SNST, Simple Nutritional Screening Tool; Spinal NST, Spinal Nutritional Screening Tool; SPSM, Short Portable Sarcopenia Measure; 3-MinNS, 3-Minute Nutrition Screening; ♦, tool assessed for and found to be valid; X, tool assessed for and found not to be valid; –, tool not assessed for/not enough information provided.

Malnutrition

Nineteen screening tools were found to be validated for the assessment of malnutrition. However, only 12 of these incorporated a question about dietary intake or decline. Six measured percentage weight loss over time, and 13 assessed BMI. In particular, those tools that had high sensitivities and specificities (Malnutrition Screening Tool for Cancer and Spinal Nutrition Screening Tool) did not encompass all parts of the agreed-upon definition. The Spinal Nutrition Screening Tool did not assess BMI and the Malnutrition Screening Tool for Cancer made no assessment of quantifying weight loss within a specified time frame. The 3-MinNS was the tool that incorporated the consensus definition criteria and also had high sensitivities and specificities (>80%).

DISCUSSION

Overview

Although current systematic reviews have described the results of studies examining malnutrition screening tools, to our knowledge this is the first review to examine tools that have been validated against another to assess cachexia, sarcopenia, and malnutrition. There has only been one previous review on tools for cachexia, sarcopenia, and malnutrition (53). It did not include psychometric evaluation, did not comment on the validity of the tools, or compare them to the agreed-upon consensus definitions. Existing systematic reviews of malnutrition screening tools have been limited to describing tools that are non–disease-specific and "quick and easy" or that have been narrative in nature.

Thirty-eight studies describing 22 tools were identified and judged for validity against a reference method. In the absence of a generally recognized gold standard for screening, assessment by a professional, DXA, CT, MRI, anthropometric measures, or the screening tools SGA and MNA were considered "valid" reference methods by our research group (13, 44–46). Although cross-sectional imaging is now used routinely for body-composition analysis, only 3 tools identified were validated against CT. The heterogeneity in populations, age groups, tools, and reference methods was large, and therefore pooling of results was impossible. Most tools had only been tested in one population, making the drawing of any definitive conclusions difficult. There were too few disease-specific tools to conclude which would be superior for different disease processes.

TABLE 5
Sensitivity, specificity, predictive values, and reproducibility of the studies included[1]

Study, year (reference)	Screening tool	Sensitivity, %	Specificity, %	PPV	NPV	Agreement
Woo et al., 2014 (15)	SARC-F	3.8–9.9	94.2–99.1	8.4–54.8	78.4–94.9	0.78–0.90
Miller et al., 2009 (16)	SPSM	—	—	—	—	—
Argiles et al., 2017 (17)	CASCO	—	—	—	—	—
Weekes et al., 2004 (18)	BAPEN	—	—	—	—	0.77
Mirmiran et al., 2011 (19)	BNST	86.7	61.7	79.1	73.1	0.74
Laporte et al., 2015 (20)	CNST	72.6	85.1	81.2	77.0	0.88
Ignacio et al., 2005 (21)	CONUT	92.3	85	—	—	0.488
Guerra et al., 2017 (22)	EDC	17.1	98.3	89.1	58.9	0.803
Abd-El-Gawad et al., 2014 (23)	GNRI	83.1	51.2	78.95	58.33	0.713
Tammam et al., 2009 (24)	INSYST	95–100	65–83	—	—	0.73
Kim et al., 2011 (31)	MST	93	93	98.4	72.7	0.7
Ferguson et al., 1999 (25)	MST	100	92	80	100	0.83
Isenring et al., 2006 (26)	MST	67	86	—	—	0.53
Neelemaat et al., 2011 (27)	MST	49	86	—	—	0.33
Nursal et al., 2005 (28)	MST	73	55	—	—	0.28
Young et al., 2013 (29)	MST	73	70	—	—	—
Wu et al., 2012 (30)	MST	39	93	—	—	0.21
Bhuachalla et al., 2018 (31)	MST	39.4–100	47–74.6	—	—	0.71
Leiopold et al., 2018 (32)	MST	72.2	83.8	69.6	85.4	—
Kim et al., 2011 (33)	MSTC	94	84.2	67.8	97.6	0.70
Boleotome et al., 2012 (34)	MUST	80	89	100	100	—
Leistra et al., 2013 (35)	MUST	75	94	43	98	—
Sharma et al., 2017 (36)	MUST	69.7	75.8	75.4	70.1	0.49
Neelemaat et al., 2011 (27)	MUST	96	80	—	—	—
Kyle et al., 2006 (37)	MUST	61	79	—	—	—
Young et al., 2013 (29)	MUST	87	86	—	—	—
Almeida et al., 2012 (38)	MUST	85	93	—	—	—
Velasco et al, 2011 (39)	MUST	72	90	—	—	—
Bhuachalla et al., 2018 (31)	MUST	20.8–72.8	48–98.3	—	—	0.816
Prasad et al., 2012 (40)	NRI	92.9	32.39	80.41	60.53	0.63
Faramarzi et al., 2013 (41)	NRI	66	60	64	62	0.267
Bhuachalla et al., 2018 (31)	NRI	21.2–95	21.2–92.1	—	—	—
Neelemaat et al., 2011 (27)	NRS-2002	92	85	—	—	—
Kyle et al., 2006 (37)	NRS-2002	62	93	—	—	—
Young et al., 2013 (29)	NRS-2002	90	83	—	—	—
Almeida et al., 2012 (38)	NRS-2002	80	89	—	—	—
Bauer et al., 2005 (42)	NRS-2002	70	85	—	—	—
Velasco et al., 2011 (39)	NRS-2002	74	87	—	—	—
Soderhamn et al., 2002 (43)	NUFFE	71	86	—	—	—
Xia et al., 2016 (51)	R-NST	97.3	74.4	88.0	93.6	0.95
Duerksen et al., 2000 (44)	SGA	—	—	—	—	—
Cooper et al., 2002 (45)	SGA	59–68	61–65	41–42	70–83	0.6
Moriana et al., 2014 (46)	SGA	—	—	—	—	—
Kruizenga et al., 2005 (47)	SNAQ	79	83	70	89	—
Leistra et al., 2013 (35)	SNAQ	43	99	78	96	—
Harada et al., 2017 (48)	SNAQ	43	99	—	—	—
Neelemaat et al., 2011 (27)	SNAQ	75	84	—	—	—
Young et al., 2013 (29)	SNAQ	79	90	—	—	—
Susetyowati et al., 2014 (49)	SNST	97	80	78	92	—
Wong et al., 2011 (50)	Spinal NST	85.7	76.1	62	92	0.57
Lim et al., 2009 (52)	3-MinNS	86	83	67	94	—

[1]BAPEN, British Association for Parenteral and Enteral Nutrition; BNST, British Nutrition Screening Tool; CASCO, Cachexia score; CNST, Canadian Nutrition Screening Tool; CONUT, Controlling Nutritional Status; EDC, European Society for Clinical Nutrition and Metabolism Diagnostic Criteria for Malnutrition; GNRI, Geriatric Nutrition Risk Index; INSYST, Imperial Nutritional Screening System; MST, Malnutrition Screening Tool; MSTC, Malnutrition Screening Tool for Cancer; MUST, Malnutrition Universal Screening Tool; NPV, negative predictive value; NRI, Nutritional Risk Index; NRS-2002, Nutritional Risk Screening; NUFFE, Nutritional Form for the Elderly; PPV, positive predictive value; R-NST, Renal Nutritional Screening Tool; SARC-F, Strength, Assistance with walking, Rise from a chair, Climb stairs, and Falls; SGA, Subjective Global Assessment; SNAQ, Short Nutritional Assessment Questionnaire; SNST, Simple Nutritional Screening Tool; Spinal NST, Spinal Nutritional Screening Tool; SPSM, Short Portable Sarcopenia Measure; 3-MinNS, 3-Minute Nutrition Screening.

Problems with current screening tools

For the generalized adult population, all of the tools showed inconsistent results with regard to their validity. The SGA, which is often considered to be the industry standard (54) and against which many tools are validated, has not itself been well validated. It performed well against the diagnostic criteria but sensitivities and specificities were either not recorded or poor. Arguably, the most well-known tools—MUST and NRS-2002—showed a variation in results from poor to good (27, 29, 34–39, 42), and consistency between groups in which the tools were studied was poor. The less well-known Nutritional Form for the Elderly (NUFFE) showed good validity, but it has been described in only a small portion of the literature and is

TABLE 6
Domains assessed by tools to measure relevant variables required to identify risks of malnutrition, sarcopenia, and cachexia[1]

	Patient reported weight loss																				
	Weight loss								Nutritional intake				Assessment of muscle mass and function		Disease state		Measures of metabolic derangement				
Screening tool	Quantified within specified time frame	Quantified without time frame	Unquantified with time frame	Unquantified, without time frame	UWL specified	Muscle mass	BMI and FFMI measurements: BMI	BMI and FFMI measurements: FFMI	Loss of appetite	Poor dietary intake/intake decline	Supplemental feeding in use?	Symptoms that would prevent eating (e.g., vomiting, ulcers)	Physical performance	Muscle strength	Presence of illness	Fatigue	Increased inflammatory markers	Anaemia	Low serum albumin	Other blood tests (e.g., glucose/urea)	QOL
Sarcopenia																					
SARC-F	X	X	X	X	X	X	X	X	X	X	X	X	✓	✓	X	X	X	X	X	X	X
SPSM	X	X	X	X	X	X	✓	✓	X	X	X	X	✓	✓	X	X	X	X	X	X	X
Cachexia																					
CASCO	X	✓	X	X	✓	X	X	✓	✓	✓	X	X	✓	X	X	✓	✓	✓	✓	✓	✓
Malnutrition																					
BAPEN	X	X	X	✓	✓	X	✓	X	X	✓	X	X	X	X	X	X	X	X	X	X	X
BNST	X	X	X	✓	✓	X	✓	X	X	✓	X	X	X	X	X	X	X	X	X	X	X
CNST	X	X	✓	X	✓	X	X	X	X	✓	X	X	X	X	X	X	X	X	X	X	X
CONUT	X	X	X	X	X	X	✓	X	X	X	X	X	X	X	✓	X	X	X	✓	✓	X
EDC	X	X	✓	X	X	X	✓	✓	X	✓	X	X	X	X	X	X	X	X	X	X	X
GNRI	X	X	✓	X	✓	X	✓	X	X	X	X	X	X	X	X	X	X	X	✓	X	X
INSYST	✓	X	X	X	✓	X	X	X	X	✓	X	X	X	X	✓	X	X	X	X	X	X
MST	X	✓	X	X	✓	X	X	X	✓	X	X	X	X	X	X	X	X	X	X	X	X
MSTC	X	X	X	✓	✓	X	✓	X	X	✓	X	X	✓	X	X	✓	X	X	X	X	X
MUST	✓	X	X	X	✓	X	✓	X	X	X	X	X	X	X	✓	X	X	X	X	X	X
NRI	X	X	✓	X	✓	X	X	X	X	X	X	X	X	X	X	X	X	X	✓	X	X
NRS-2002	X	✓	X	X	X	X	✓	X	X	✓	X	X	X	X	✓	X	X	X	X	X	X
NUFFE	X	X	X	✓	✓	X	✓	X	X	✓	X	X	X	X	X	X	X	X	X	X	X
R-NST	✓	X	X	X	X	X	X	X	✓	✓	X	✓	X	X	✓	X	X	X	X	X	X
SGA	✓	X	X	X	✓	X	✓	✓	✓	✓	✓	X	✓	X	X	X	X	X	X	X	X
SNAQ	✓	X	X	X	✓	X	✓	X	✓	✓	X	X	X	X	X	X	X	X	X	X	X
SNST	X	X	✓	X	✓	X	X	X	X	✓	X	X	X	X	X	X	X	X	X	X	X
Spinal NST	X	X	X	✓	✓	X	✓	X	✓	✓	X	X	X	X	✓	X	X	X	X	X	X
3-MinNS	✓	X	X	X	✓	X	✓	X	X	✓			X	X	✓	X	X	X	✓	X	X

[1] Data adapted from reference 51. BAPEN, British Association for Parenteral and Enteral Nutrition; BNST, British Nutrition Screening Tool; CASCO, Cachexia Score; CNST, Canadian Nutrition Screening Tool; CONUT, Controlling Nutritional Status; EDC, European Society for Clinical Nutrition and Metabolism Diagnostic Criteria for Malnutrition; FFM, fat-free mass; FFMI, fat-free mass index; GNRI, Geriatric Nutrition Risk Index; INSYST, Imperial Nutritional Screening System; MST, Malnutrition Screening Tool; MSTC, Malnutrition Screening Tool for Cancer; MUST, Malnutrition Universal Screening Tool; NRI, Nutritional Risk Index; NRS-2002, Nutritional Risk Screening; NUFFE, Nutritional Form for the Elderly; QOL, quality of life; R-NST, Renal Nutritional Screening Tool; SARC-F, Strength, Assistance with walking, Rise from a chair, Climb stairs, and Falls; SGA, Subjective Global Assessment; SNAQ, Short Nutritional Assessment Questionnaire; SNST, Simple Nutritional Screening Tool; Spinal NST, Spinal Nutritional Screening Tool; SPSM, Short Portable Sarcopenia Measure; UWL, unintentional weight loss; 3-MinNS, 3-Minute Nutrition Screening; ✓, tool assesses criteria; X, tool does not assess criteria.

TABLE 7
Newcastle-Ottawa scale adapted for cross-sectional studies[1]

	Woo (15)	Miller (16)	Anglés (17)	Weekes (18)	Mimiran (19)	Laporte (20)	Iglesias (21)	Guerra (22)	Abd-El-Dayem (23)	Tamman (24)	Ferguson (25)	Isenring (26)	Nefesmaa (27)	Nurse (28)	Young (29)	Bhuachalla (31)	Leopold (32)	Wu (30)	Kim (33)	Bolen-Tuite (34)	Lalara (35)	Shamma (36)	Kyle (37)	Almeida (38)	Velasco (39)	Prasad (40)	Faramarzi (41)	Bauer (42)	Soderhamn (43)	Durdiken (44)	Cooper (45)	Mohsina (46)	Kruizenga (47)	Harada (48)	Susetyowati (49)	Wong (50)	Xia (51)	Lim (52)
Selection (maximum: 5 stars)																																					
1. Representativeness of the sample	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
2. Sample size justified			*									*												*							*		*	*	*		
3. Response rate satisfactory	*	*	*	*	*	*			*			*	*	*	*	*	*	*			*			*	*		*	*	*	*	*	*	*	*	*	*	*
4. Ascertainment of exposure (validated measurement tool used)	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Comparability (maximum: 2 stars)																																					
1. The study controls for disease severity	*	*	*					*												*									*								
2. Study controls for other confounding factors	*	*							*										*				*										*				
Outcome (maximum: 3 stars)																																					
1. Assessment of outcome	*				*	*	*			*	*	*				*		*			*	*							*	*							
2. Appropriate statistical test described	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Total (out of 10)	7	6	5	5	6	6	6	5	6	6	6	6	4	5	4	7	4	7	5	4	4	6	6	5	6	5	4	5	5	7	7	6	4	6	4	5	5

[1]Scores <5 indicate high risk of bias. * Assessed in study and found to be present.

not implemented widely (43). The "quick and easy" screening tools, including the Short Nutritional Assessment Questionnaire and Malnutrition Screening Tool, performed reasonably well (sensitivities of ~80%) in most studies in which they were used (25–30, 35, 47, 48). Of note, because these tools are quick, they require a further detailed assessment by a qualified health professional if screening is positive. They also miss ~20% of at-risk patients at initial screening and therefore may be more useful in screening high-risk patients.

The tool that performed the best for malnutrition was the 3-MinNS (52). It showed high sensitivity and specificity (>80%) and accurately encompassed the correct diagnostic criteria (percentage of weight loss over a specified time and measurement of BMI) for malnutrition. It was validated in acute medical and surgical patients and proved quick and easy to complete. It has only been validated in one study, and therefore it cannot be assumed that it would perform as well in different patient populations. Both tools that assessed sarcopenia (SPSM, SARC-F) scored well against the agreed-upon definition (15, 16). However, the SPSM required transport of equipment and the SARC-F had a very low sensitivity (13, 15). The CASCO was the only validated screening tool for cachexia (17). It performed well against diagnostic criteria, but sensitivities and specificities were not recorded. It has also only been validated in the cancer setting; more work would be needed to validate the tool in other cachectic populations or the general adult population.

Most tools were validated in the adult hospital inpatient setting. Tools for sarcopenia have only been validated in healthy, community-dwelling elderly individuals (15, 16). Length of hospital stay is diminishing worldwide, and outpatient nutritional screening is advocated to detect patients at risk. In this review, we identified 8 studies in which outpatients were included. More studies focusing on the construct and predictive validity of tools for outpatient screening are warranted, especially because care is shifting to this setting.

The tool that appeared to have the broadest coverage was the CASCO (17). It is the only tool that screens for cachexia but also detects many of the variables required for a diagnosis of malnutrition. However, assessment of muscle mass or function (required for sarcopenia) is not included. One previous review showed that 20 screening tools appeared to be relevant for starvation, but none contained all of the currently accepted components needed to screen for sarcopenia and cachexia risk (53). Our study supports this finding.

Outlook and recommendations for future tools

A screening tool needs to be developed that encompasses the criteria to detect all 3 possible syndromes. This concept is supported by the notion that, in humans, there may be no "pure" phenotype of cachexia, because it is usually associated with reduced food intake (potential for malnutrition) and increasing age (increasing sarcopenia) (55). There is also currently a lack of agreement as to the diagnostic criteria for each syndrome and the relative importance of body-composition analysis and the nature of depleted tissue within each definition. We hypothesized that the overlap between syndromes could be shown, as in **Figure 2**, along with the identified best-performing tools for each aspect.

There are clearly many existing validated screening tools (at least for malnutrition). It is unlikely that any further novel tools will be devised without breakthroughs in biomarker development. We therefore suggest that the ideal composite tool should incorporate a stepwise assessment of nutritional status, oral intake, disease status, patient age, muscle mass and function, and metabolic derangement. The presence of underlying disease is a key question in order to stratify the syndromes. Suggested components for use in creating a new tool are depicted in **Table 8**.

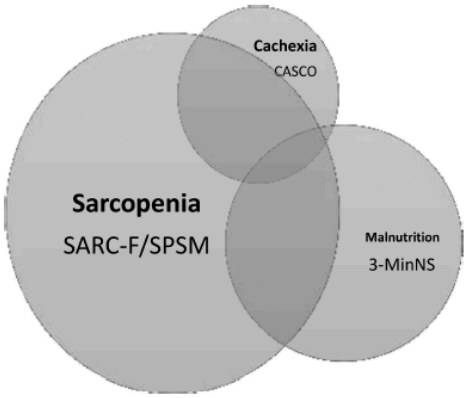

FIGURE 2 Diagram to show overlap between cachexia, sarcopenia, and malnutrition. The sizes of the circles represent the perceived sizes of each clinical problem. CASCO, Cachexia score; SARC-F, Strength, Assistance with walking, Rise from a chair, Climb stairs, and Falls; SPSM, Short Portable Sarcopenia Measure; 3-MinNS, 3-Minute Nutrition Score.

The use of screening for all 3 syndromes will allow for a more targeted intervention. Screening for cachexia, sarcopenia, or malnutrition is not warranted unless it is accompanied by an intervening care plan. It would be expected that an adequate intervention would prevent any further decline in health status and therefore lead to a positive effect on disease outcome. Most studies did not comment on intervention, which, depending on the balance of the 3 syndromes, may need to include varying attention to nutrition, exercise, and measures to combat inflammation.

Strengths, limitations, and assessment of bias

One of the strengths of this review is that it provides a complete overview of tools that have been validated for cachexia, sarcopenia, and malnutrition. The review used the consensus definitions of each syndrome. We are aware, however, that many

TABLE 8
Suggested components for use in creating a new screening tool

1. Quantification of weight loss
2. Measurement of BMI
3. Assessment of appetite/dietary intake and decline
4. Underlying health state: Is there the presence of chronic disease?
5. Take into account patient's age (i.e., age >60 y more likely to be sarcopenic)
6. Assessment of muscle mass and function
7. Measurement of metabolic derangement/increased C-reactive protein

other definitions exist. However, there were a number of study limitations. We did not describe reliability, repeatability or other clinical outcome measures in any great detail. There was a risk of bias when assessing each tool for their predictive validity. Studies may have been biased if they did not adjust for factors such as cancer stage or disease severity. As clinical outcome is affected by more than just nutritional status alone, adjusting for these variables is important. Nutritional intervention is likely to improve outcomes for malnutrition, but potentially not for age-related sarcopenia or established cachexia. Only one study discussed whether they did this. There is no agreed-upon "gold standard" tool, and therefore we chose cross-sectional imaging and the SGA and MNA on the basis of the results of previous studies (13). Tools that were compared with potentially less-valid standards were also included to allow a wider analysis. Full nutrition assessments were different in each study, ranging from anthropometric to biochemical measures and full assessment by a medical professional. Conclusions from this review were based on the original studies in which there may have been varying definitions with regard to the subject group, syndrome, or assessment undertaken. Another potential limitation is that we excluded modified versions of pre-existing tools. They were excluded because reliability and validity data would only relate to the modified tool and it was therefore difficult to assess improvements from the original. It is possible that these tools were being improved or evaluated more thoroughly.

Conclusions

We have highlighted that many practitioners who regularly come into contact with patients suffering from weight loss are not able to easily screen between the conditions of cachexia, sarcopenia, and malnutrition because there is no single validated tool that can be implemented for the assessment of all 3 conditions. The adaptation of existing screening tools incorporating all relevant criteria described in this review would be optimal for diagnosis and to direct the content of complex interventions.

The authors' responsibilities were as follows— JM, RJES, MJJ, and LW: designed the project; JM and UN: conducted the review; JM and LW: wrote the manuscript; RJES, DC, and MJJ: critically appraised the manuscript; MJJ and RJES: had overall responsibility for the final content; and all authors: read and approved the final manuscript. The authors declared no conflicts of interest.

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Neuromuscular junctions are stable in patients with cancer cachexia

Ines Boehm, … , Ross A. Jones, Thomas H. Gillingwater

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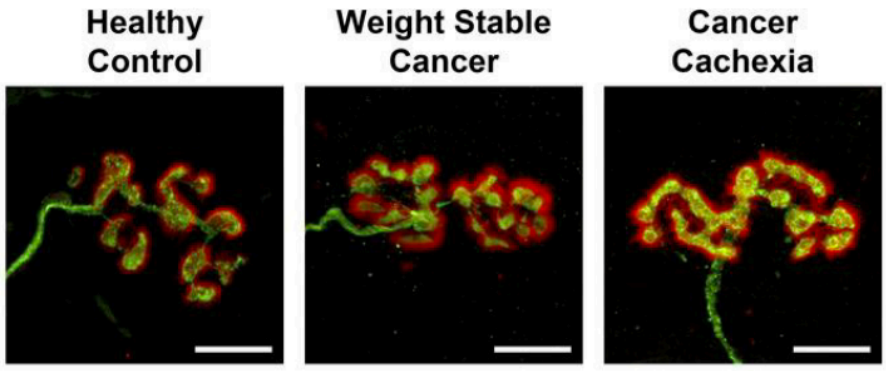
Concise Communication | Neuroscience | Oncology

Graphical abstract



Neuromuscular junctions (NMJs) are stable in patients with cancer cachexia

Neuromuscular junctions are stable in patients with cancer cachexia

Ines Boehm,[1] Janice Miller,[2] Thomas M. Wishart,[3] Stephen J. Wigmore,[2] Richard J.E. Skipworth,[2] Ross A. Jones,[1] and Thomas H. Gillingwater[1]

[1]Biomedical Sciences, Edinburgh Medical School, Edinburgh, United Kingdom. [2]Clinical Surgery, Edinburgh Medical School and Royal Infirmary of Edinburgh, Edinburgh, United Kingdom. [3]Roslin Institute, University of Edinburgh, Easter Bush Campus, Midlothian, United Kingdom.

Cancer cachexia is a major cause of patient morbidity and mortality, with no efficacious treatment or management strategy. Despite cachexia sharing pathophysiological features with a number of neuromuscular wasting conditions, including age-related sarcopenia, the mechanisms underlying cachexia remain poorly understood. Studies of related conditions suggest that pathological targeting of the neuromuscular junction (NMJ) may play a key role in cachexia, but this has yet to be investigated in human patients. Here, high-resolution morphological analyses were undertaken on NMJs of rectus abdominis obtained from patients undergoing upper GI cancer surgery compared with controls ($N = 30$; $n = 1,165$ NMJs). Cancer patients included those with cachexia and weight-stable disease. Despite the low skeletal muscle index and significant muscle fiber atrophy ($P < 0.0001$) in patients with cachexia, NMJ morphology was fully conserved. No significant differences were observed in any of the pre- and postsynaptic variables measured. We conclude that NMJs remain structurally intact in rectus abdominis in both cancer and cachexia, suggesting that denervation of skeletal muscle is not a major driver of pathogenesis. The absence of NMJ pathology is in stark contrast to what is found in related conditions, such as age-related sarcopenia, and supports the hypothesis that intrinsic changes within skeletal muscle, independent of any changes in motor neurons, represent the primary locus of neuromuscular pathology in cancer cachexia.

Introduction

Cachexia is a severe and debilitating syndrome, commonly associated with cancer and characterized by the loss of muscle with or without corresponding loss of adipose tissue (1). Cancer cachexia is a major burden for both patients and health care systems globally, with profoundly negative impacts on the response to treatment, quality of life, and long-term survival of patients (2). At present, the consensus definition of cancer cachexia confirms loss of skeletal muscle as a key feature of the condition (3), largely mediated by proinflammatory cytokines and tumor-associated mediators, resulting in the activation of catabolic pathways in skeletal muscle (4). In this regard, cancer cachexia shares many of the muscle-specific and systemic inflammatory pathways common to the muscular dystrophies (5, 6).

Although the majority of research to date has focused on muscle abnormalities as the major locus of pathophysiology in cachexia, many lines of evidence have implicated the neuromuscular junction (NMJ) as a critical and early mediator of neuromuscular dysfunction and breakdown. Principally, NMJ dysfunction and denervation represent a shared hallmark of several related muscle-wasting conditions and neuromuscular diseases (7–9). For example, NMJ pathology is considered to represent a key early driver of neuromuscular defects in age-related sarcopenia (10–12), at least in part the result of an age-related loss of motor neurons (13). As cancer cachexia and sarcopenia share similar molecular mechanisms (14), and as cachexia is considered to be a multifactorial syndrome that includes components of both age-related sarcopenia and bed rest/reduced physical activity (15), the NMJ has similarly been implicated in the pathogenesis of cachexia. The identification of displaced mononuclei in muscle of cachectic patients and C26 tumor-bearing mice has been used to suggest the presence of denervation (16). Furthermore, a member of the ubiquitin-proteasome pathway (MuRF1) pivotal to muscle wasting in tumor-bearing mice (and other murine models of muscle wasting) is critical for maintenance of the NMJ (17). Additionally, recent mouse studies of mTOR signaling, a key regulator of protein synthesis that is suppressed by inflammatory mediators in cancer cachexia (18), have shown that muscle-specific deletion of mTOR or Raptor results in muscle fibrillation and NMJ fragmentation (19).

The role of the nerve-muscle interface in human cachexia is therefore of interest to both clinician and basic scientists and relevant to our understanding of disease pathophysiology and the development of effective treatments. Thus, cancer cachexia has been the subject of coculture/informatics projects (20) and ongoing patient studies. Experimental evidence supporting a direct role for NMJs in human cachexia is, however, highly reliant on studies of animal models of related conditions (21–23). Importantly, recent data have revealed striking and unexpected differences between the cellular and molecular anatomy of human NMJs

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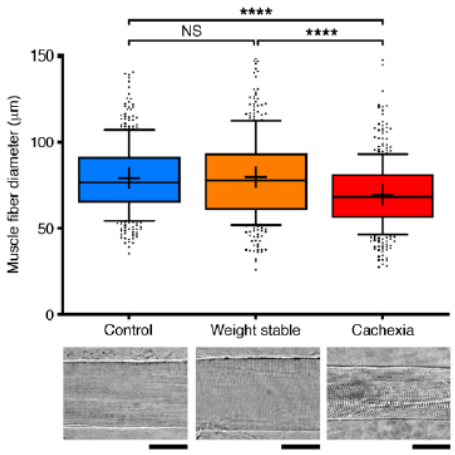

Figure 1. Atrophy of skeletal muscle fibers in cancer cachexia. Upper panel shows a box and whisker plot of muscle fiber diameters in control (n = 10 patients), weight-stable (n = 10), and cachectic (n = 10) patients. Bottom panels show representative micrographs of single, teased muscle fibers from control (left), weight-stable (middle), and cachectic (right) patients. Scale bars: 50 μm. Cachectic patients had significantly reduced muscle fiber diameters compared with weight-stable and control cases (control, n = 388; weight stable, n = 362; cachexia, n= 400 muscle fibers). Boxes contain the mean (+) and median (line) muscle-fiber diameters for the group and enclose the central 25th-75th percentile of the data, and whiskers extend from the 10th-90th percentile. Outlying data points are shown beyond the whiskers. ****P < 0.0001, 1-way ANOVA paired with Tukey's post-hoc test. Individual P values are shown in Supplemental Table 2.

compared with those of other model organisms (24), suggesting that findings from animal models may not be directly applicable to human patients.

The present study builds upon our recent work establishing a robust protocol to facilitate the sampling and high-resolution, quantitative morphological analyses of human NMJs from patients undergoing surgery (24, 25). We have adapted these protocols to facilitate a comprehensive analysis of the NMJ in patients with cancer cachexia.

Results and Discussion

To investigate the role of the NMJ in human cancer cachexia, we performed a comprehensive morphometric analysis of the NMJ in samples of rectus abdominis (RA) muscle obtained from patients undergoing surgery for upper gastrointestinal (GI) cancer (Supplemental Table 1; supplemental material available online with this article; https://doi.org/10.1172/JCI128411DS1). RA was selected for 2 reasons: (a) it is readily accessible in the majority of surgical approaches to the abdomen and therefore a well-utilized muscle for sampling and characterization of human cancer cachexia (26) and (b) nerve roots innervating RA are unlikely to be affected by radiculopathy or other common spinal pathology, rendering neurogenic remodeling an unlikely possibility.

Cachectic patients demonstrated significantly lower skeletal muscle index (SMI) by computerized tomography (CT) (Supplemental Figure 1) criteria compared with weight stable patients (Supplemental Table 1) and also demonstrated a trend toward lower subcutaneous adiposity and higher visceral adiposity. These 2 body composition phenomena are associated with worsened outcomes in cancer patients (27–29), further confirming cachectic patients as a high-risk group. Two patients in the weight-stable cancer group exhibited a small degree of weight loss, but were not cachectic by the consensus definition (3, 30).

To confirm/validate the patient groupings based on the clinical and radiological guidelines (percentage of weight loss and SMI, respectively), we assessed muscle fiber diameter on teased muscle fiber preparations from RA. Mean muscle fiber diameter was significantly reduced (by almost 15%) in the cachectic patients compared with both control and weight-stable groups (P < 0.0001; Figure 1). However, there was no significant difference in mean muscle fiber diameter between control patients and those with weight-stable disease (P > 0.05; Figure 1). These observations are in keeping with published data showing marked muscle fiber atrophy in cachexia (31, 32) and support the patient group allocations based on consensus definition (3).

Given that muscle fiber atrophy would be predicted to result from and/or lead to NMJ instability based on findings from animal studies (8, 9), we next performed an initial qualitative assessment of NMJs. Despite the presence of muscle fiber atrophy in cachectic patients, NMJ morphology was indistinguishable from that observed in weight stable and control patients (Figure 2). NMJs of all 3 cohorts were noted to display the typical "nummular" morphology characteristic of human NMJs (24) and, despite the predicted heterogeneity in form across the complete pool of NMJs (Figure 3), we found no evidence of gross pathological changes or denervation of skeletal muscle fibers.

Although initial qualitative observations suggested an absence of gross pathology at the NMJ, more subtle changes in NMJ morphology could still have been present in the cachectic patients. We therefore undertook a comprehensive NMJ-morph analysis of all patient NMJs (Figure 3 and Supplemental Table 2). NMJs from RA were initially compared with our existing database of human NMJs from several lower limb muscles (24) to determine their likeness (or otherwise) to established human NMJ morphology in other muscle groups. NMJ-morph analysis revealed comparable NMJ morphology across RA and 4 lower limb muscles (Supplemental Figure 2).

Quantitative NMJ-morph analyses confirmed that there were no significant differences in any aspect of NMJ morphology in RA across the 3 patient groups (Figure 3 and Supplemental Table 2). Crucially, and in stark contrast to predictions based on mechanistic animal models, there was no evidence of denervation (defined by percentage overlap [Figure 3C] between nerve terminal and endplate), demonstrating that this is not a major feature of pathogenesis in cachectic patients. Similarly, there was no evidence for increased NMJ fragmentation (Figure 3D), a classical feature of NMJ pathology found in animal models of neurodegeneration (7–9) and cardiac cachexia (33).

Alongside analyses of denervation and NMJ fragmentation, our NMJ-morph analysis confirmed no statistically significant

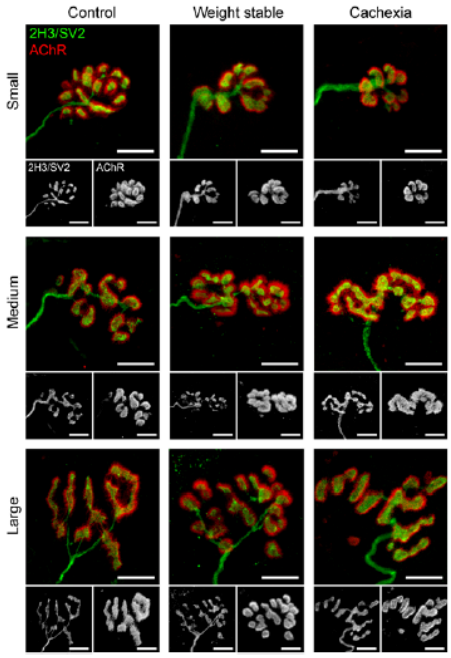

Figure 2. Conservation of NMJ morphology in cancer cachexia. Confocal micrographs of representative small, medium, and large NMJs from RA in the 3 patient groups. Despite heterogeneity in size and shape of individual NMJs, overall morphology was conserved across all groups, with no evidence of NMJ pathology in either the cachexia or weight-stable groups. Axon and nerve terminals are shown in green (2H3/SV2) and AChRs of the motor endplate in red (α-BTX). Scale bars: 10 μm.

changes in any of the other morphological variables investigated (Figure 3 and Supplemental Table 2); similar axon diameters (Figure 3A) (approximately 1 μm) were observed across all 3 groups, with no evidence of axonal swelling, neurofilament accumulation, or polyneuronal innervation (indicative of denervation/reinnervation processes). Thus, neither gross nor subtle perturbations at the NMJ were observed in cachectic patients. However, the relative contribution of muscle regeneration and myopathic changes still requires definitive demonstration in human cachexia patients.

It should be noted that cachectic patients in the current study represent the more extreme end of the clinical diagnostic definition criteria, having both weight loss and low CT muscularity. However, our study only enrolled patients who were eligible for surgery with potentially curative intent. It is not possible, therefore, to draw conclusions concerning a possible late disruption of the NMJ in palliative cancer patients with refractory cachexia and severe functional impairment.

While RA proved to be an excellent muscle for the current study, our findings differed from those observed in human age-related sarcopenia and in animal models of muscle wasting, both situations in which weight-bearing muscles are usually assessed experimentally. It remains possible therefore that skeletal muscle with different functional and/or biochemical properties may respond differently in cachexia. Equally, the observed differences between human patients and animal models may reflect anticipated differences in cachexia pathophysiology between the two. Human cachexia is proposed to be a multifactorial condition in which diverse drivers of muscle wasting all contribute to varying degrees in individual patients and tumor types (15). In comparison, in vivo tumor-bearing models may demonstrate accelerated wasting, which lacks the complexity and heterogeneity of the human condition. This supposition is supported by previous studies that have demonstrated little overlap in gene expression profiles between muscle biopsies from human cancer patients and equivalent animal models (34).

All patients received intravenous atracurium besilate during anesthesia, which competitively displaces acetylcholine from its receptors. Its half-life is 17 to 21 minutes, but whether it has longer lasting effects on the form or function of the NMJ is not known (35). Importantly therefore, samples from the current study were compared with lower limb samples from patients who had received spinal anesthesia only. No differences were observed, suggesting that the choice of anesthetic was unlikely to have had any significant impact on the morphology of the NMJ.

In summary, we report that the human NMJ retains full structural integrity in both cachexia and weight-stable cancer. This suggests that denervation of skeletal muscle and/or NMJ disruption are not major drivers of disease pathogenesis in cancer cachexia and that cancer cachexia represents a unique neuromuscular condition that needs to be differentiated from related conditions, including age-related sarcopenia. This observation supports the hypothesis that intrinsic changes within skeletal muscle, independent of any changes in motor neurons, represent the primary locus of pathology in cachexia. Since the NMJ remains intact in patients with cancer cachexia, promotion of muscle hypertrophy using exercise and neural stimulation should remain a viable therapeutic intervention for future clinical trials.

Methods

Patient recruitment. Patients with a confirmed diagnosis of GI cancer suitable for surgical resection with curative intent were recruited from the regional multidisciplinary team meeting (n = 20; Supplemental Table 1) and included patients with both cancer cachexia (n = 10) and weight-stable disease (n = 10) based on consensus definition (3). Suitable age-matched control patients undergoing a range of elective abdominal procedures (e.g., repair of aortic aneurysm or donor nephrectomy) were also recruited (n = 10; Supplemental Table 1). All patients were at least 18 years of age and provided written, informed consent (inclusion criteria). Patients with a previous history of malignancy were not eligible for the control cohort (exclusion criteria).

Body composition analysis. See Supplemental Methods.

Tissue sampling. Tissue sampling was performed under general anesthesia at the start of the surgical procedure. If patients underwent neoadjuvant chemotherapy, surgery was performed 4 to 6 weeks following cessation of treatment. Biopsies of RA muscle were obtained following initial opening of the abdomen. RA is a well-characterized tissue for the study of human cancer cachexia, for which the results of previous studies have been reviewed recently (26). Evident changes

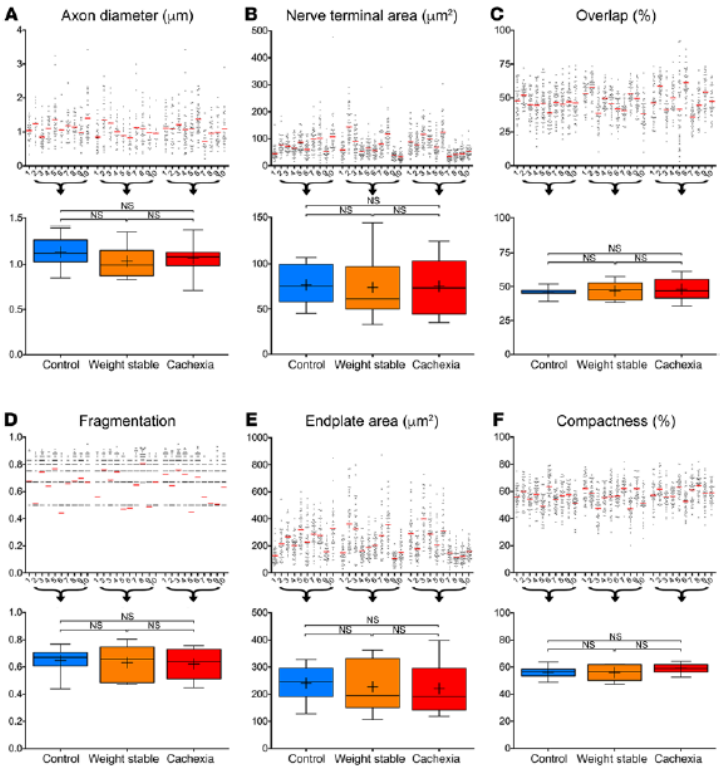

Figure 3. Structural integrity of the NMJ in cancer cachexia. Morphometric analysis using NMJ-morph revealed that NMJ morphology is conserved in both cachexia and weight-stable disease. Data presented as a pair of charts (scatterplot, above; box and whisker plot, below) for key NMJ variables, including measurements of axon diameter (**A**) and pre- and postsynaptic architecture (**B**–**F**). Scatterplots depict the approximately 40 individual NMJs (data points) for the 10 patients (1 to 10) in each group; the mean NMJ value is given by the red line; the observed heterogeneity is a normal feature of human NMJ morphology. Box and whisker plots constructed using the mean patient data in each group (10 patients; control NMJs, n = 387; weight stable NMJs, n = 386; cachexia NMJs, n = 392). Boxes contain the mean (+) and median (line) values for each NMJ variable and enclose the central 25th-75th percentile of the data; whiskers represent the maximum and minimum values. One-way ANOVA paired with Tukey's post hoc test.

of wasting, including fiber atrophy, have been robustly and repeatedly observed in several studies (32). RA has an "in-series" architecture, with motor endplate bands located throughout its length (36). To ensure successful sampling, a longitudinal strip of muscle lying between 2 contiguous tendinous intersections was obtained using sharp dissection (approximately 0.5 × 0.5 × 2.0 cm). Samples were immediately fixed in 4% paraformaldehyde (PFA) for approximately 2 hours, followed by washing and storage in 1× PBS.

Tissue processing and NMJ immunohistochemistry. See Supplemental Methods.

Confocal imaging & NMJ-morph analysis. See Supplemental Methods.

Statistical analysis. Statistical analyses were performed using GraphPad Prism 6 software. Results are expressed as mean (± SEM). Group comparison of normally distributed data (based on D'Agostino-Pearson test) was performed by 1-way ANOVA and Tukey's post hoc test; nonparametric data were analyzed by Kruskal-Wallis test and Dunn's post hoc test. $P < 0.05$ was considered significant.

Study approval. Ethics approval for tissue sampling was granted by the National Health Service Lothian Ethics Committee (IRAS 190214) in accordance with the Helsinki Declaration. All patients were at least 18 years of age and provided written informed consent.

Author contributions

All authors designed the experiments and drafted the manuscript. IB, JM, RJES, RAJ, and THG performed the studies and analyzed the data. Authorship order was agreed on according to the time and effort committed to the project.

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Address correspondence to: Thomas H. Gillingwater, Old Medical School (Anatomy), University of Edinburgh, Teviot Place, Edinburgh, EH8 9AG, United Kingdom. Phone: 44.131.650.3724; Email: t.gillingwater@ed.ac.uk.

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Supplemental Information for Boehm et al.

Supplementary Figure S1

Figure S1. Cross sectional CT image at L3 showing demarcation of skeletal muscle and adipose tissue using Slice-o-matic and ABACS software.

Red = Skeletal Muscle, Green = Intramuscular fat, Yellow = Visceral adipose tissue, Blue = Subcutaneous adipose tissue

Supplementary Figure S2

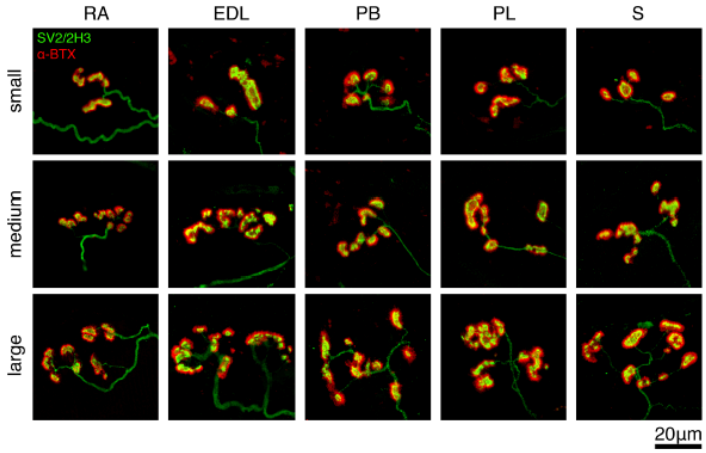

Figure S2. Comparative anatomy of human NMJs in *rectus abdominis* (RA), *extensor digitorum longus* (EDL), *peroneus brevis* (PB), *peroneus longus* (PL) and *soleus* (S)

No major differences in NMJ morphology were observed between lower limb muscles *extensor digitorum longus* (EDL, n = 10 patients), *peroneus brevis* (PB, n = 10 patients), *peroneus longus* (PL, n = 10 patients), *soleus* (S, n = 10 patients) and *rectus abdominis* (RA, n = 6 patients). The lower limb data of 10 patients from the previously published data set in Jones et al 2017 was chosen for its completeness of pre- and post-synaptic parameters. SV2/2H3 = synaptic vesicle 2 and neurofilament (presynapse, green); α-BTX = α-bungarotoxin (acetylecholine receptors, red). Scale bar = 20 µm. Box and whisker plots show the mean (± SEM) as a '+' over the median.

Supplementary Table S1

Patient Group (n = 10)	Ctrl	Weight Stable	Cachexia	F (DFn, DFd) or Kruskal Wallis statistic	P	Mean Difference Ctrl vs Weight Stable	Mean Difference Ctrl vs Cachexia	Mean Difference Weight Stable vs Cachexia
Male:Female	9:1	7:3	8:2	/	/	/	/	/
Age (years)	64 ± 2.89 (min 49, max 81)	66 ± 3.89 (min 40, max 83)	68 ± 3.00 (min 49, max 78)	F (2, 27) = 0.3887	0.682	-1.90	-4.10	-2.20
BMI	28.68 ± 1.32 (min 20.0, max 36.2)	27.00 ± 1.78 (min 22.4, max 41.1)	25.69 ± 1.13 (min 20.4, max 31.8)	F (2, 27) = 1.096	0.349	1.69	3.00	1.31
% weight loss	0 % ± 0 % (min 0 %, max 0 %)	0.89 % ± 0.5 % (min 0 %, max 4.38 %)	7.99 % ± 1.65 % (min 2.70 %, max 19.1 %)	22.71	< 0.0001 ****	-3.400 %	-16.10 % ****	-12.70 % **
SMI	52.0 ± 2.08 (min 38.5, max 59.6)	56.9 ± 4.0 (min 43.7, max 78.5)	40.3 ± 1.75 (min 30.6, max 52.3)	12.98	0.0015 **	-2.100	11.10 *	13.20 **
VATI	64.4 ± 9.85 (min 15.8, max 111.6)	53.0 ± 16.6 (min 6.73, max 188.4)	73.7 ± 11.5 (min 33.5, max 134.0)	F (2, 27) = 0.644	0.5329	11.39	-9.342	-20.73
SATI	67.3 ± 14.0 (min 2.58, max 153.2)	60.3 ± 11.3 (min 18.32, max 132.3)	55.7 ± 4.53 (min 39.1, max 82.1)	0.281	0.8688	1.900	1.700	-0.2000
Cancer type: Oesophageal ACC Oesophageal SCC Oesophageal undifferentiated Gastric ACC Colonic ACC	/	4 1 0 4 1	7 1 1 1 0	/	/	/	/	/
ECOG	/	0 (min 0, max 1)	0 (min 0, max 1)	/	/	/	/	/

Table S1. Demographics of GI cancer patients recruited for NMJ analysis. All data are mean and standard error of the mean (SEM), except for ECOG performance score, which is represented as median with range. Statistical significance for age and BMI was determined using a one-way ANOVA with a Tukey's post-hoc test. Statistical significance for % weight loss, SMI and SATI was determined using a Kruskall-Wallis test with a Dunn's post-hoc test. BMI = body mass index; SMI = skeletal muscle index VATI = visceral adipose tissue index; SATI = subcutaneous adipose tissue index; ECOG = Eastern Cooperative Oncology Group performance status, ACC = adenocarcinoma, SCC = squamous cell carcinoma.

Supplementary Table S2

	Ctrl n = 10 387 NMJs	Weight Stable n = 10 386 NMJs	Cachexia n = 10 392 NMJs	F (DFn, DFd) or Kruskal Wallis statistic	P	Mean Difference Ctrl vs Weight Stable	Mean Difference Ctrl vs Cachexia	Mean Difference Weight Stable vs Cachexia
core variables								
pre-synaptic								
1) Nerve Terminal Area (µm²)	76.53 ± 6.84	73.70 ± 11.08	74.86 ± 10.02	F (2, 27) = 0.023	0.978	2.832	1.669	-1.163
2) Nerve Terminal Perimeter (µm)	143.22 ± 9.88	136.16 ± 13.49	134.90 ± 11.67	F (2, 27) = 0.145	0.866	7.061	8.318	1.257
3) Number of Terminal Branches	37.88 ± 3.21	37.01 ± 2.56	37.42 ± 2.47	0.008	0.996	-0.250	-0.350	-0.100
4) Number of Branch Points	17.29 ± 3.01	17.39 ± 1.98	17.66 ± 1.94	F (2, 27) = 0.007	0.994	-0.097	-0.097	-0.272
5) Total Length of Branches (µm)	64.16 ± 6.08	61.25 ± 6.98	60.02 ± 5.99	F (2, 27) = 0.112	0.895	2.916	4.148	1.232
post-synaptic								
6) AChR Area (µm²)	129.92 ± 9.66	121.98 ± 14.53	124.80 ± 15.01	F (2, 27) = 0.092	0.913	7.935	5.117	-2.818
7) AChR Perimeter (µm)	150.94 ± 9.48	154.28 ± 15.64	153.86 ± 18.94	F (2, 27) = 0.014	0.986	-3.338	-2.922	0.416
8) Endplate Area (µm²)	241.02 ± 21.06	228.18 ± 29.65	221.47 ± 29.81	F (2, 27) = 0.134	0.875	12.840	19.550	6.711
9) Endplate Perimeter (µm)	77.84 ± 4.28	77.42 ± 6.36	77.32 ± 6.67	F (2, 27) = 0.002	0.998	0.421	0.520	0.099
10) Endplate Diameter (µm)	27.30 ± 1.32	28.39 ± 2.35	27.99 ± 2.25	F (2, 27) = 0.074	0.929	-1.093	-0.690	0.403
11) Number of AChR Clusters	4.15 ± 0.28	4.12 ± 0.42	4.28 ± 0.46	F (2, 27) = 0.045	0.956	0.023	-0.131	-0.154
derived variables								
pre-synaptic								
12) Average Length of Branches (µm)	1.91 ± 0.11	1.78 ± 0.15	1.78 ± 0.10	F (2, 27) = 0.415	0.665	0.132	0.135	0.003
13) Complexity	4.35 ± 0.15	4.34 ± 0.12	4.37 ± 0.12	F (2, 27) = 0.011	0.989	0.011	-0.016	-0.027
post-synaptic								
14) Average Area of AChR Clusters (µm²)	42.32 ± 4.65	37.65 ± 3.64	38.87 ± 2.83	F (2, 27) = 0.410	0.667	4.669	3.448	-1.221
15) Fragmentation	0.65 ± 0.032	0.63 ± 0.04	0.62 ± 0.03	F (2, 27) = 0.132	0.877	0.016	0.025	0.009
16) Compactness (%)	56.08 ± 1.32	55.97 ± 1.79	59.01 ± 1.13	F (2, 27) = 1.439	0.255	0.108	-2.939	-3.047
17) Overlap (%)	46.01 ± 0.9986	46.84 ± 2.13	48.18 ± 2.54	F (2, 27) = 0.301	0.742	-0.836	-2.173	-1.337
18) Area of Synaptic Contact (µm²)	58.94 ± 4.56	57.02 ± 8.30	58.07 ± 7.36	F (2, 27) = 0.019	0.981	1.919	0.866	-1.053
associated nerve & muscle variables								
19) Axon Diameter (µm)	1.14 ± 0.06	1.03 ± 0.06	1.07 ± 0.06	2.178	0.337	5.800	3.200	-2.600
20) Muscle Fibre Diameter (µm)	79.82 ± 1.25	79.11 ± 1.02	70.44 ± 1.06	F (2, 1132) = 22.350	< 0.0001	0.714	9.383 ****	8.669 ****
21) Number of Axonal Inputs	1.08 ± 0.03	1.08 ± 0.04	1.03 ± 0.10	0.239	0.888	1.650	0.000	-1.650

Table S2. Overview of complete NMJ-morph data.

The mean values ± SEM are listed for each patient group, representing the mean of the entire analysed NMJ data set. Statistical significance from a one-way ANOVA paired with a Tukey's post-hoc test. Statistical significance of average area of AChR clusters and fragmentation determined via Kruskal Wallis non-parametric test, followed by a Dunn's post-hoc test.

Supplementary Methods

Body composition analysis

Skeletal muscle index (SMI) was calculated either from routine staging CT scans performed prior to surgical intervention, or the post chemotherapy re-staging scan if the patients underwent neoadjuvant chemotherapy (n = 5 in the cachectic group and n = 3 in the weight stable group). Digital CT images obtained with a spiral CT scanner were analysed using Slice-O-matic and ABACS software (Supplementary Figure 1). SMI was derived from measurements of muscle cross-sectional area normalized to body stature (cm^2/m^2) at the level of the 3rd lumbar vertebra (L3) (Supplementary Figure 1). SMI cut-offs for low SMI were obtained from previously published reference data (30). Cachexia was defined according to standard consensus (>2% weight loss and low muscularity (3) as this definition has been shown to demonstrate histological muscle wasting in previous studies (32).

Tissue processing and NMJ immunohistochemistry

Teased muscle fibre preparations were immunohistochemically labelled for NMJ analysis as previously described (24) to visualise key pre-synaptic proteins (the synaptic vesicle protein SV2 and the neurofilament protein 2H3), with post-synaptic acetylcholine receptors (AChRs) labelled using tetramethylrhodamine-conjugated alpha-bungarotoxin. Optimal labelling was achieved with incubation in primary antibodies for 3 nights and secondary antibodies for 1 night. Samples were whole-mounted in Mowiol and stored at -20ºC prior to imaging.

Primary antibodies: mouse monoclonal SV2 and 2H3 (1:50; Developmental Studies Hybridoma Bank, University of Iowa). Secondary antibodies: AlexaFluor-488-conjugated donkey anti-mouse IgG (H+L) antibody (1:400; A21202, Life Technologies). Tetramethylrhodamine-conjugated alpha-bungarotoxin (2 µg/mL; BTIU00012, VWR International).

SV2 and 2H3 antibodies, developed by Buckley, K.M. and Jessell, T.M. / Dodd, J. respectively, were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242.

Confocal imaging & NMJ-morph analysis

Confocal images of individual en face NMJs and their pre-terminal axons were acquired on a Zeiss LSM 710 or Nikon A1R confocal laser-scanning microscope, using standardised imaging approaches (24,25). In addition, individual muscle fibre diameter measurements were obtained on an Olympus IX71 microscope and Hamamatsu C4742-95 camera with Openlab Improvision software (Jones et al. 2016; Jones et al. 2017). All images were then analysed according to a standardised workflow ('NMJ-morph'), as previously described (24,25). NMJ-morph data output generates 21 separate morphological descriptors for each NMJ (see Supplementary Table 2), including pre- and post-synaptic variables (e.g. nerve terminal area, motor endplate area, etc.), derived variables (e.g. nerve terminal complexity, motor endplate fragmentation, etc.) and related nerve and muscle measurements (including motor axon diameter and muscle fibre diameter). As per NMJ-morph guidelines, a total of 40 NMJs were analysed (where possible) for each individual patient/muscle sample (N = 30 patients) giving a complete dataset of n = 1,165 NMJs.

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Author for correspondence:
Ross A. Jones
e-mail: ross.jones@ed.ac.uk

†These authors contributed equally to this study.

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aNMJ-morph: a simple macro for rapid analysis of neuromuscular junction morphology

Gavin Minty[1,2,†], Alex Hoppen[3,†], Ines Boehm[1,2,†], Abrar Alhindi[1,2], Larissa Gibb[1,2], Ellie Potter[1,2], Boris C. Wagner[1,2], Janice Miller[4], Richard J. E. Skipworth[4], Thomas H. Gillingwater[1,2] and Ross A. Jones[1,2]

[1]Edinburgh Medical School: Biomedical Sciences (Anatomy), and [2]Euan MacDonald Centre for Motor Neurone Disease Research, University of Edinburgh, Edinburgh EH8 9AG, UK
[3]RWTH Aachen University, 52062 Aachen, Germany
[4]Clinical Surgery, University of Edinburgh, Royal Infirmary of Edinburgh, Edinburgh, EH16 4SA, UK

IB, 0000-0002-9343-0944; THG, 0000-0002-0306-5577; RAJ, 0000-0002-1569-2500

Large-scale data analysis of synaptic morphology is becoming increasingly important to the field of neurobiological research (e.g. 'connectomics'). In particular, a detailed knowledge of neuromuscular junction (NMJ) morphology has proven to be important for understanding the form and function of synapses in both health and disease. The recent introduction of a standardized approach to the morphometric analysis of the NMJ—'NMJ-morph'—has provided the first common software platform with which to analyse and integrate NMJ data from different research laboratories. Here, we describe the design and development of a novel macro—'automated NMJ-morph' or 'aNMJ-morph'—to update and streamline the original NMJ-morph methodology. ImageJ macro language was used to encode the complete NMJ-morph workflow into seven navigation windows that generate robust data for 19 individual pre-/post-synaptic variables. The aNMJ-morph scripting was first validated against reference data generated by the parent workflow to confirm data reproducibility. aNMJ-morph was then compared with the parent workflow in large-scale data analysis of original NMJ images (240 NMJs) by multiple independent investigators. aNMJ-morph conferred a fourfold increase in data acquisition rate compared with the parent workflow, with average analysis times reduced to approximately 1 min per NMJ. Strong concordance was demonstrated between the two approaches for all 19 morphological variables, confirming

the robust nature of aNMJ-morph. aNMJ-morph is a freely available and easy-to-use macro for the rapid and robust analysis of NMJ morphology and offers significant improvements in data acquisition and learning curve compared to the original NMJ-morph workflow.

1. Background

Synaptic connectivity is central to the structure and functioning of the mammalian nervous system. In practice, the detailed analysis of synaptic connectivity—'connectomics'—remains a formidable task. Even in small laboratory animals (e.g. mice, rats) that are routinely used to model human disease, the cerebral cortex may contain up to 1700 synaptic connections per 1500 μm^3 volume [1]. Given the complexity of the central nervous system, the study of 'model synapses' has been critical to the progress of synaptic biology, with the neuromuscular junction (NMJ)—the synapse between lower motor neuron and skeletal muscle fibre—representing the paradigm example.

The importance of normal synaptic connectivity is evidenced by the multitude of neurodegenerative conditions that are underpinned by synaptic dysfunction and/or degeneration at the NMJ. For example, myasthenia gravis and its related syndromes, along with motor neuron diseases such as amyotrophic lateral sclerosis and spinal muscular atrophy, all demonstrate varying degrees of synaptic pathology at the NMJ as either a cause or consequence of the underlying disease mechanism [2–5]. Finding effective treatments for these conditions ultimately depends on a greater understanding of the normal and pathological architecture of mammalian synapses, including NMJs.

Until recently, however, even basic quantification of the gross cellular anatomy of the NMJ has been hampered by the lack of a standardized approach to morphometric analysis. Following the introduction of NMJ-morph in 2016 [6]—a simple but robust method for NMJ quantification—a growing number of research groups have now used this approach to gain important insights into a diverse range of conditions and species [7–12]. For example, NMJ-morph was pivotal to the first major study on the cellular and molecular anatomy of the human NMJ [7]. Here, NMJ-morph revealed in detail the unique 'nummular' morphology of the human NMJ and further demonstrated its structural stability over the lifespan, in direct contrast to age-related fragmentation of rodent NMJs. The sensitivity of NMJ-morph analysis has identified subtle changes in NMJ morphology found in Charcot–Marie–Tooth disease [8] and helped characterize NMJ degeneration in CHCHD10-encoded mitochondrial myopathy associated with motor neuron disease [9]. Most recently, NMJ-morph has been used in the study of human pathology, revealing that NMJs are stable in patients with cancer cachexia—the severe loss of skeletal muscle that is commonly associated with many forms of cancer [12].

At present, the two major barriers to more widespread adoption of NMJ-morph are its associated learning curve, and relatively low data throughput in real time (approx. 12 NMJs h^{-1}). Here, we present a macro update to the original NMJ-morph workflow—'automated NMJ-morph' or 'aNMJ-morph'—to streamline and expedite data acquisition.

2. Results and discussion

To support the continued uptake of NMJ-morph in the field of synaptic biology and related disciplines, we developed a macro update of the original workflow—'aNMJ-morph'. The full version of the macro (compatible with both Windows and Mac operating systems) and supporting materials (including tutorial videos, sample images and reference spreadsheets) is freely available for download at Edinburgh DataShare [13]. For a complete understanding of the individual morphological variables (and their derivations), we recommend that users of aNMJ-morph are familiar and competent with the use of the original workflow in a practical setting [6].

The standard NMJ-morph workflow uses ImageJ/Fiji [14] and the Binary Connectivity [15] plugin (all in the public domain) to generate data for 19 different morphological variables on confocal images of individual NMJs [6]. For each image, this workflow requires the user to navigate through approximately 75 separate drop-down menus in Fiji, followed by manual input of raw data into a spreadsheet proforma (containing formulae for generating additional derived variables). The initial published estimate of throughput suggested a work rate of approximately 30 NMJs h^{-1} for an experienced user [6]; in practice, we have found that most users are able to analyse approximately 12 NMJs h^{-1} (approx. 5 min per NMJ), though

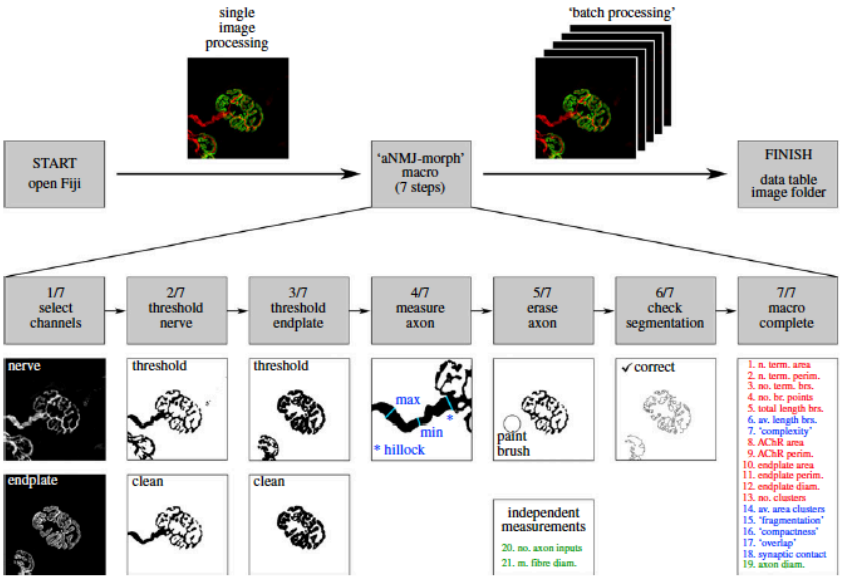

Figure 1. 'aNMJ-morph'. The 'aNMJ-morph' macro comprises seven instruction windows that guide the user through the various stages of image analysis, and can be used for either single image or batch processing. The only manual inputs include image thresholding, axon processing (measure/erase) and confirmation of image segmentation. At completion, aNMJ-morph generates a data table containing 19 individual morphological variables, corresponding to those of the original NMJ-morph workflow; the 'number of axonal inputs' and 'muscle fibre diameter' are measured independently. 'Core variables' are shown in red typeface, 'derived variables' in blue and 'associated nerve and muscle variables' in green. Note: For single NMJ analysis, first open the image, then select the macro from the plugins. For batch processing, first open the macro, then select the image folder; the macro will automatically cycle through each image in turn to completion.

the throughput can be increased by the use of keyboard shortcuts built-in to ImageJ, and by the assignment of additional shortcuts.

In comparison, aNMJ-morph (figure 1) streamlines the workflow into seven instruction windows and tabulates the results automatically (in the form of a .csv spreadsheet), reducing the image acquisition time to just under 1.5 min per NMJ. In addition to the substantial time saving, one of the major advantages of aNMJ-morph is the simplicity of data acquisition. This enables the user to focus on the most critical step—accurate image thresholding—and reduces common errors resulting from data transcription and transposition [6].

To screen for any unanticipated scripting and/or technical issues arising during the development of the macro, a reference dataset (of 40 NMJs) was first analysed by a single investigator using both the original (NMJ-morph) and automated (aNMJ-morph) workflows. For this exercise, the same threshold settings were selected for both manual and automated assessments. As expected, the results of this initial validation generated near perfect correlations ($r = 0.954$–1.000; all $p < 0.0001$; table 1).

The fractionally lower correlation coefficients for the pre-synaptic variables ($r = 0.954$–0.998; table 1) compared to the post-synaptic variables are a consequence of the manual erasing of nerve terminal axon following axonal diameter measurement. Manual input also accounted for the minor differences in endplate diameter between the two methods ($r = 0.992$; table 1 and figure 2*e*). For the number of AChR clusters ($r = 0.986$; table 1) and its derivations (fragmentation and average area of AChR clusters), the discrepancy between NMJ-morph and aNMJ-morph was methodological (figure 2*d*). In 3 of 40 endplates, the 'fill holes' function in the macro reduced the total number of clusters in each NMJ by one, due to the enclosure of a single AChR cluster within another on the segmented image (figure 2*d*). These occasional examples were not found to have any statistically significant effect in practice (see below).

To assess the usability of aNMJ-morph in practice, two pairs of investigators were then tasked with analysing a large volume of new NMJ images (figure 3 and table 1) using either the original NMJ-morph

Table 1. NMJ-morph (manual) versus aNMJ-morph (macro). Correlation coefficients (*r*) comparing the two methods of image analysis for each variable. During the development of aNMJ-morph, a single investigator applied the two approaches using the same threshold settings ($n = 40$ NMJs; *Within User*). After validation, two pairs of investigators worked in real time on a large image bank using either aNMJ-morph or the original workflow ($n = 240$ NMJs; *Between User*). Correlation coefficients support the robust nature of the aNMJ-morph macro in a practical setting. Correlation coefficients (*r*) are Pearson for parametric variables, Spearman for non-parametric variables; $p < 0.0001$ for all correlation coefficients.

	NMJ-morph (manual) versus aNMJ-morph (macro)	
morphological variable	*Within User (r)*	*Between User (r)*
pre-synaptic		
(1) nerve terminal area (μm^2)	0.998	0.892
(2) nerve terminal perimeter (μm)	0.994	0.875
(3) number of terminal branches	0.978	0.762
(4) number of branch points	0.987	0.740
(5) total length of branches (μm)	0.977	0.791
(6) average length of branches (μm)	0.954	0.661
(7) 'complexity'	0.978	0.785
post-synaptic		
(8) AChR area (μm^2)	1.000	0.923
(9) AChR perimeter (μm)	1.000	0.858
(10) endplate area (μm^2)	1.000	0.982
(11) endplate perimeter (μm)	1.000	0.949
(12) endplate diameter (μm)	0.992	0.891
(13) number of AChR clusters	0.986	0.937
(14) average area of AChR clusters (μm^2)	0.971	0.823
(15) 'fragmentation'	0.986	0.936
(16) 'compactness' (%)	1.000	0.827
(17) 'overlap' (%)	1.000	0.765
(18) area of synaptic contact (μm^2)	1.000	0.914
associated nerve and muscle		
(19) axon diameter (μm)	0.960	0.758

workflow or the macro. Images were obtained from ongoing research projects and included NMJs from a range of both slow and fast-twitch muscles (e.g. soleus and extensor digitorum longus, respectively; $n = 240$ NMJs in total). In addition, the new images were of a different file format (.nd2, Nikon) to those used for the initial macro development (.lsm, Zeiss; see Methods) and each investigator used a different workstation and operating system (to ensure compatibility with both Windows and Mac).

As before, correlation analyses revealed strong concordance between the two approaches (NMJ-morph versus aNMJ-morph) for all variables ($r = 0.661$–0.982; all $p < 0.0001$; table 1). The greater range of correlation coefficients highlights the normal inter-user variability that is expected in relation to thresholding and manual data input, and is in keeping with the variability described in the original NMJ-morph workflow [6]. Crucially, in relation to the counting of AChR clusters, correlations were strong between the two methods ($r = 0.937$, $p < 0.0001$; table 1), supporting this approach in the automation process (figure 2*d*).

Of particular note, aNMJ-morph conferred a fourfold increase in data acquisition rate, with average analysis time per NMJ reduced from nearly 5.5 min (319 s) to just over 1 min (79 s). In practical terms, this represents a substantial improvement in work rate and potential throughput. To enable robust comparison of different NMJ populations (e.g. muscles, animals, species, etc.) we recommend datasets of at least 30–40 NMJs per sample based on NMJ-morph guidelines [6]; in real time, complete NMJ datasets can now be obtained in just over half an hour with aNMJ-morph, compared to around

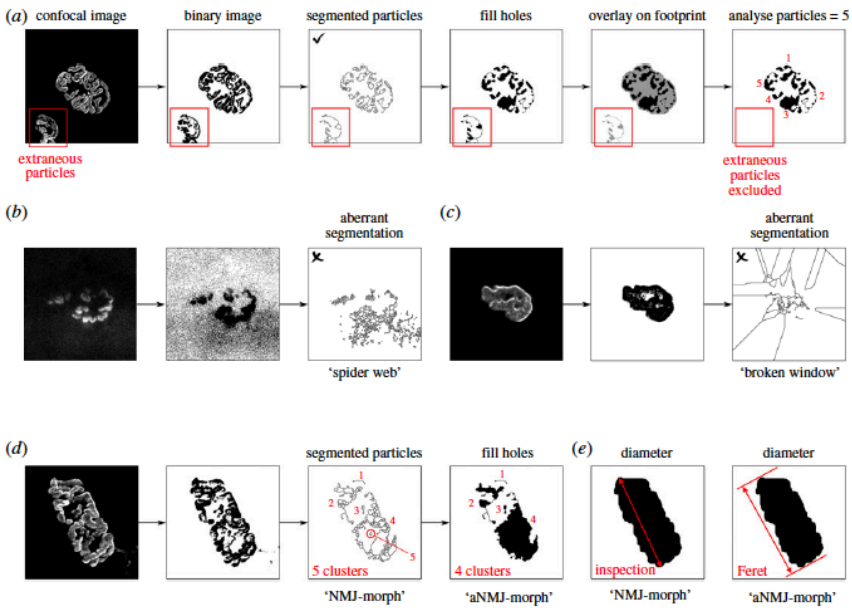

Figure 2. Automation within aNMJ-morph. Several processes within the original NMJ-morph workflow required additional scripting to enable full automation. (*a*) Automated counting of AChR clusters necessitated the exclusion of extraneous background particles. (*b*,*c*) Examples of aberrant image segmentation. These images are identified at the 'check segmentation' step of aNMJ-morph (window 6/7; figure 1). (*d*) Variation in particle number between NMJ-morph and aNMJ-morph (in this example, five clusters versus four clusters); in practice, these occasional examples of spurious counting were not found to be statistically significant. (*e*) Automation of endplate diameter measurement using the Feret's diameter function in ImageJ/Fiji.

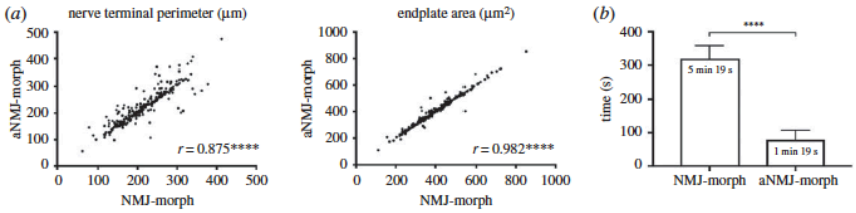

Figure 3. NMJ-morph (manual) versus aNMJ-morph (macro). aNMJ-morph offers a robust and expeditious alternative to the original NMJ-morph workflow. Two pairs of investigators analysed a large image bank ($n = 240$ NMJs) using either aNMJ-morph or the original workflow. (*a*) Correlation analyses demonstrated strong concordance between the two methods for all variables; examples of pre- and post-synaptic variables are illustrated (nerve terminal perimeter and endplate area). (*b*) aNMJ-morph conferred a fourfold reduction in analysis time (approx. 1 min per image) compared with the original workflow (approx. 5 min per image). Pearson correlation; $^{****}p < 0.0001$.

3 h or so previously, depending on the level of proficiency with NMJ-morph. In addition, aNMJ-morph eliminates the common errors associated with manual data transfer via an automatically curated .csv file containing the 19 morphometric variables (and additional information on image size and threshold selection).

We anticipate that other research groups will now wish to trial the macro in different settings, e.g. with NMJ images acquired using different scanning parameters and/or file types. To support these adaptations, we recommend that users first validate the macro output against equivalent data generated using the original workflow [6] to confirm the functionality of the macro in different settings. We also encourage the development of machine-learning algorithms based on the existing NMJ-morph approach to further refine and improve the rate of data acquisition.

3. Conclusion

'Automated NMJ-morph'—'aNMJ-morph'—is a freely available update to the existing NMJ-morph workflow, for the rapid and robust analysis of NMJ morphology. aNMJ-morph offers significant advantages over the original workflow, with a clear emphasis on accessibility and ease of use. It is hoped that aNMJ-morph will be of particular interest to NMJ biologists and associated researchers who are engaged in large-scale data analysis of comparative NMJ morphology.

4. Methods

4.1. Macro scripting and validation

A Fiji/ImageJ-based macro (automated NMJ-morph or aNMJ-morph) was first scripted using ImageJ macro language (IJM) [16] to encode the complete NMJ-morph workflow as described in the original manuscript [6]. The full IJM-text transcription is included in electronic supplementary material, file S1. The final aNMJ-morph macro comprises seven instruction windows and generates a spreadsheet containing data for 19 individual pre-/post-synaptic variables (figure 1).

For the majority of operations in the NMJ-morph workflow, the IJM scripting involved straightforward coding of the correct sequence of drop-down menus and checkbox selections within Fiji. Several operations required further development to enable full automation, including the 'number of AChR clusters' and 'endplate diameter' (figure 2).

Assessment of the 'number of AChR clusters' in the original NMJ-morph workflow involved manual counting of 'segmented particles' (figure 2*a*, panels 1–3) in order to distinguish genuine clusters (i.e. those contributing to the endplate) from extraneous particles (e.g. adjacent endplates or background noise). For aNMJ-morph, we were able to automate this process via several additional steps in the macro scripting (figure 2*a*, panels 4–6; electronic supplementary material, file S1). These steps involved overlaying filled particles onto the footprint of the endplate (using the 'fill holes' and 'concatenate' functions). Automated counting (analyse particles) then returned the number of particles lying within the footprint alone (i.e. those contributing to the endplate) while excluding any extraneous particles.

On rare occasions (less than 1 in 1000 images), the use of the 'segmented particles' function in the original NMJ-morph workflow resulted in spurious fragmentation of the endplate, with images resembling 'spider webs' or 'broken windows' (figures 2*b*,*c*). In our experience, this was usually the result of poor image quality from the outset (figure 2*b*); as per the original guidelines [6], we recommend that these NMJs are excluded entirely. In exceptional circumstances, aberrant segmentation is noted in images of sound quality (figure 2*c*); in these instances, automated counting of clusters is not possible (measurement of other variables is unaffected, e.g. area, perimeter, etc.). To address these eventualities in the macro, an instruction window was incorporated prompting users to confirm appropriate segmentation of the image (figure 1, window 6/7; electronic supplementary material, file S1); in circumstances of abnormal segmentation, the macro will still measure and record the other variables.

The measurement of 'endplate diameter' was the only other variable that required automation. In the original NMJ-morph workflow, the maximum linear dimension of the endplate was judged on inspection and recorded manually. In the macro, this value was obtained automatically by using the 'Feret's diameter' function in ImageJ, which provides an analogous measurement (figure 2*e*; electronic supplementary material, file S1).

The only manual aspects of the original NMJ-morph workflow to be retained in the macro related to image thresholding and axon processing (figure 1, windows 1–5/7). Accurate image thresholding is critical to the generation of robust NMJ-morph data [6] and it was crucial to retain this step under user-defined control; the original NMJ-morph manuscript [6] should be consulted for detailed instruction/discussion of image thresholding. Of note, thresholded binary images must be compared to the original raw images to confirm accurate image reproduction. Similarly, the measurement of axon diameter requires a degree of user-dependent decision-making that is not compatible with simple automation, particularly in relation to NMJs of certain species, e.g. human NMJs [7,12].

Two further variables are conventionally recorded as part of a complete NMJ-morph analysis—'number of axonal inputs' and 'muscle fibre diameter'. Since both variables require independent measurement, they were not suitable for automation. Polyinnervation (i.e. number of axonal inputs > 1) only occurs in certain specific circumstances (e.g. development, pathology) and requires careful assessment, while muscle fibre diameter is measured on a separate set of images [6].

During development, aNMJ-morph was compared against the original workflow by a single investigator using the same image threshold settings. To assess the usability of aNMJ-morph in a wider context (different investigators, different laboratories, etc.), four different investigators trialled the two methods on a much larger image bank (see Results).

4.2. NMJ images and file types

All NMJ images used in the development and testing of the aNMJ-morph macro were obtained from previous and/or ongoing animal research projects covered by the requisite personal and project licences granted by the UK Home Office. All images were captured using Zeiss/Nikon confocal microscopes, with the file types .lsm/.nd2, respectively. The macro uses the maximum intensity projection of the corresponding *z*-stack and has been validated for these file types and image formats only. For all other file types, we recommend that users first validate the macro output against equivalent data generated with the original workflow [6] before proceeding.

4.3. ImageJ/Fiji and binary connectivity

The macro was developed using ImageJ/Fiji software (v.: 2.0.0-rc-67/1.52i / build: 1762a07c5c). The latest version of ImageJ/Fiji is freely available at https://fiji.sc [14] including instructions for download. The Binary Connectivity plugin is freely available at https://blog.bham.ac.uk/intellimic/g-landini-software [15] under the section 'Morphological Operators for ImageJ' (including instructions for installation). To manage updates, the latest version of the macro will be hosted at Edinburgh DataShare [13].

4.4. Statistical analysis

All statistical analyses were performed on GraphPad Prism software; individual statistical tests are indicated in the relevant figure legends.

Data accessibility. The aNMJ-morph macro, tutorial video, sample images and reference spreadsheets are available for download at Edinburgh DataShare: https://doi.org/10.7488/ds/2625 [13].

Authors' contributions. G.M., A.H., I.B. and R.A.J. developed the aNMJ-morph macro. G.M., A.H., I.B., A.A., L.G., E.P., B.C.W., T.H.G. and R.A.J. performed all experiments and data analysis. All authors drafted and approved the manuscript for submission.

Competing interests. The authors have no competing interests to declare.

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ORIGINAL ARTICLE

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Adipose depot gene expression and intelectin-1 in the metabolic response to cancer and cachexia

Janice Miller[1], Gillian Dreczkowski[2], Michael I. Ramage[1], Stephen J. Wigmore[1], al-name()='id']/@value)","^JCSM|^RCO2|^CRT2")=1?^!"number(boolean(ancestor::node()[local-name()='subComponent']))"[?tpacr=0]>Iain J. Gallagher[2] & Richard J.E. Skipworth[1*]

[1]*Clinical Surgery, Royal Infirmary of Edinburgh, University of Edinburgh, Edinburgh, UK,* [2]*Faculty of Health Science and Sport, University of Stirling, Stirling, UK*

Abstract

Background Cancer cachexia is a poorly understood metabolic consequence of cancer. During cachexia, different adipose depots demonstrate differential wasting rates. Animal models suggest adipose tissue may be a key driver of muscle wasting through fat–muscle crosstalk, but human studies in this area are lacking. We performed global gene expression profiling of visceral (VAT) and subcutaneous (SAT) adipose from weight stable and cachectic cancer patients and healthy controls.

Methods Cachexia was defined as >2% weight loss plus low computed tomography-muscularity. Biopsies of SAT and VAT were taken from patients undergoing resection for oesophago-gastric cancer, and healthy controls (n = 16 and 8 respectively). RNA was isolated and reverse transcribed. cDNA was hybridised to the Affymetrix Clariom S microarray and data analysed using R/Bioconductor. Differential expression of genes was assessed using empirical Bayes and moderated-t-statistic approaches. Category enrichment analysis was used with a tissue-specific background to examine the biological context of differentially expressed genes. Selected differentially regulated genes were validated by qPCR. Enzyme-linked immunosorbent assay (ELISA) for intelectin-1 was performed on all VAT samples. The previously-described cohort plus 12 additional patients from each group also had plasma I = intelectin-1 ELISA carried out.

Results In VAT vs. SAT comparisons, there were 2101, 1722, and 1659 significantly regulated genes in the cachectic, weight stable, and control groups, respectively. There were 2200 significantly regulated genes from VAT in cachectic patients compared with controls. Genes involving inflammation were enriched in cancer and control VAT vs. SAT, although different genes contributed to enrichment in each group. Energy metabolism, fat browning (e.g. uncoupling protein 1), and adipogenesis genes were down-regulated in cancer VAT (P = 0.043, $P = 5.4 \times 10^{-6}$ and $P = 1 \times 10^{-6}$ respectively). The gene showing the largest difference in expression was ITLN1, the gene that encodes for intelectin-1 (false discovery rate-corrected P = 0.0001), a novel adipocytokine associated with weight loss in other contexts.

Conclusions SAT and VAT have unique gene expression signatures in cancer and cachexia. VAT is metabolically active in cancer, and intelectin-1 may be a target for therapeutic manipulation. VAT may play a fundamental role in cachexia, but the down-regulation of energy metabolism genes implies a limited role for fat browning in cachectic patients, in contrast to pre-clinical models.

Keywords Cancer cachexia; Adipose; Intelectin; Microarray; Genes

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**Correspondence to: Richard Skipworth, Clinical Surgery, Royal Infirmary of Edinburgh, 51 Little France Crescent, Edinburgh, EH16 4SA, UK.*
Email: richard.skipworth@nhslothian.scot.nhs.uk
Iain J. Gallagher and Richard J.E. Skipworth are joint senior authors.

Introduction

Cachexia is a complex metabolic syndrome associated with several chronic diseases, including cancer, that is characterised by loss of lean and adipose tissue[1]. Cachexia is a major burden for cancer patients, and it impacts negatively on their response to treatment, their quality of life, and survival. Although the consensus definition of cancer cachexia[2] emphasises loss of lean tissue (particularly skeletal muscle) as a key factor in the diagnosis of the condition, the loss of adipose tissue in cancer cachexia is increasingly thought to play an important role.[3,4]

In cancer, the loss of adipose tissue is driven by lipolysis[5] rather than adipocyte apoptosis or necrosis. Mediators of lipolysis in cancer cachexia (CC) are largely unknown. In the past, members of our group have identified increased mRNA expression of zinc-α2-glycoprotein (ZAG), a proposed lipid mobilizing factor, in fat samples from CC patients; however, serum ZAG levels were unchanged from controls.[6,7] Microarray analysis has revealed that changes in the transcriptome of subcutaneous fat in CC are opposite to those seen in obesity, underlining the importance of lipolysis.[8] Visceral adipose tissue is thought to be lost more rapidly than subcutaneous adipose tissue during cachexia, suggesting differential adipose depot-dependent responses to the wasting process though this has not been consistently demonstrated in humans.[9] Bioinformatic analysis of mRNA expression in visceral (omental) and subcutaneous adipose depots in noncachectic, obese endometrial cancer patients demonstrated 19 shared biological pathways, 18 of which were regulated in opposite directions between the fat depots.[10] Recently, focus has concentrated on the concept of 'fat–muscle crosstalk' in CC.[1] Notably, genetic ablation of lipolytic pathways in adipocytes protects against muscle mass loss in animal models of CC.[11] Inhibition of lipolysis through genetic ablation of adipose triglyceride lipase or hormone sensitive lipase was shown to reduce muscle wasting.[11] These data support the loss of visceral fat driven by lipolytic mechanisms as an early event in CC with a potential effect on skeletal muscle.

Global gene expression profiling is an effective method to examine regulatory pathways in patho-physiological contexts.[12] In our previous studies, microarray analysis of skeletal muscle biopsies taken at the time of resectional surgery for upper gastrointestinal (GI) cancer revealed an 83-gene signature that was able to identify patients with >5% weight loss.[13] In further studies, a transcriptomic comparison of sequential muscle biopsies (at surgery and 8 months post-surgery) from upper GI cancer patients revealed 1868 regulated genes associated with cancer and weight loss.[14] Contrary to expectation, the vast majority (94%) of genes were down-regulated. Category analysis of the differentially expressed genes showed that both anabolic and catabolic process gene expression was suppressed in cancer. Furthermore, there was a lack of substantive overlap with transcriptomic signatures from endurance training, strength training, ageing, or simple dieting. Highly enriched categories included lipid oxidation, fatty acid metabolism, and peroxisome pathways, implying a role for fat–muscle crosstalk in CC.[14] A better understanding of the physiological effects of cancer on adipose tissue might open new therapeutic avenues for the amelioration of both fat and skeletal muscle wasting resulting in improved outcomes.

In the current study, we examined the global transcriptome of VAT and SAT depots in patients with confirmed CC, according to the diagnostic consensus definition [>2% weight loss and low muscularity on a computed tomography (CT) scan; n = 8] compared with weight stable cancer (CWS) patients (n = 8), as well as healthy controls undergoing donor nephrectomy (n = 8). These data provide the first examination of global gene expression in these two fat depots in CC and provide a basis for hypothesis generation and exploring treatments for CC.

Methods

Study participants

Patients undergoing surgical resection with curative intent for upper GI cancer (oesophageal, gastric, or pancreatic) were recruited via the regional multidisciplinary team meeting. Healthy controls undergoing surgery for renal transplant donation were approached at their pre-assessment appointment. Participants had to be over the age of 18 and able to give written, informed consent. The study was approved by the Lothian regional ethics committee (IRAS ID: 190214) and conformed to the declaration of Helsinki. Control and CWS patients were excluded if they were found to be sarcopenic on CT scan. Patient demographics are described in terms of median (interquartile range). A Kruskal Wallis test was used to compare groups.

Adipose tissue biopsies

Following surgical skin incision under general anaesthesia, biopsies of subcutaneous fat were taken from all patients at the start of the operation. Visceral fat was sampled from the greater omentum in cancer patients and perinephric fat from the transplant donors after the kidney had been excised. All samples were snap frozen in liquid nitrogen and stored at −80°C until analysis.

Plasma samples

Venous blood samples were taken at induction of anaesthesia from all patients. Samples were centrifuged at 4°C at 15 000 *g*

for 15 min. Plasma was then withdrawn and divided into 1 mL aliquots and frozen at −80°C until use.

Muscle cross-sectional area

Skeletal muscle, visceral, and subcutaneous adipose cross-sectional areas were calculated from routine staging CT scans performed prior to any surgical intervention or neo-adjuvant chemotherapy. Digitally stored CT images completed with a spiral CT were analysed using slice-O-matic and ABACS software (Voronoi Health Analytics, Canada). The cross-sectional area for muscle, visceral, and subcutaneous adipose tissue was normalized for stature (cm^2/m^2) at the level of the third lumbar vertebrae [skeletal muscle index (SMI), visceral adipose index, and subcutaneous adipose index, respectively]. SMI cut-offs for low muscularity were taken from previous reference studies of cancer patients by Martin *et al.*[15] Cachexia was classified according to the consensus definition by Fearon *et al.* (specifically >2% weight loss with low muscularity).[2] This specific definition of CC was used as it was shown to be associated with confirmed histological muscle fibre wasting in our previous studies.[16]

Transcriptomic analysis

Aliquots of adipose tissue were homogenised in a Qiazol reagent (Qiagen, UK) using the BeadBeater instrument with 1.5 mm ceramic beads (Qiagen, UK). Tissue aliquots were shaken at 5000 Hz for 30 s with 30 s cooling on ice. This process was repeated three times or until no gross debris was visible. Samples were then spun at 2800 g for 15 mins at 4°C and the supernatant collected into fresh Eppendorf tubes. RNA was isolated from adipose tissue samples using the Qiagen RNeasy lipid tissue kit (Qiagen, UK) following the manufacturer's directions. RNA was quantified using the Denovix DS11 FX+ spectrophotometer (Denovix, UK). RNA quality was examined using the RNA iQ assay and the Qubit 4 instrument (Thermo Fisher, UK). All samples returned quality scores ≥7.7. Complementary DNA was generated using the GeneChip WT Plus Reagent kit (Thermo Fisher, UK) as per directions. Samples were hybridised to the Clariom S GeneChip microarray (Thermo Fisher, UK) as per manufacturers directions. After washing and staining, images were captured, and CEL files generated using the GeneChip Scanner 3000 (Thermofisher UK). The raw CEL files can be accessed at the Gene Expression Omnibus (GSE131835).

Quantitative polymerase chain reaction

Total RNA was extracted as above, and cDNA was prepared using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, cat no. 4368814) with 500 ng total RNA input for each reaction. Quantitative PCR (qPCR) was carried out using Luminaris Hi Green Low ROX qPCR Master Mix (Thermo Fisher UK, Cat no. K0974) 5 μL, 0.3 μL forward and reverse primer at 10 μm each and 3.4 μL ddH_2O. Primers were selected from the Primer Bank resource[17] except for actin beta and GAPDH. These latter primers were designed to span exons using the Primer-BLAST resource.[18] We selected primers to cover genes involved in inflammation (ACVR2A1, IL6, and IL18), adipogenesis (LEPR, STAT5A, and PDCD4), adipose browning (UCP1, PPARGC1A, and PRDM16) as well as genes that demonstrated a substantial statistically significant fold change by microarray data (ITLN1, ALOX15, and PPARA). We also profiled five genes to use as reference values in PCR data normalization as recommended by the MIQE guidelines.[19] PCR reactions were carried out on the LightCycler 480 Instrument with the following protocol; 50°C 2mins (uracil–DNA–glycosylase treatment), 95°C 10 min (DNA denaturation) and 40 cycles of 95°C 15 s and 60°C 60s before a final melting curve step. Samples were run in triplicate on 384-well plates. Analysis of qPCR data was carried out using the NormqPCR package in R/Bioconductor.[20] The geNorm method[21] was used to identify the four most stable control genes from actin beta, GAPDH, YWHAZ, POLR2A, and PSMB2, and the geometric mean of these was used to normalise the cycle threshold values for the remaining genes. Relative gene expression was calculated using the delta–delta Cq method (14). Primers used are shown in the Supporting Information.

Enzyme-linked immunosorbent assay

Intelectin-1 (Itln1) concentration was assessed in the VAT and plasma using an enzyme-linked immunosorbent assay [(ELISA) (Amsbio EH0564); sensitivity <46.875 pg/mL; reference range: 78.125–5000 pg/mL]. A further 36 patients were recruited to increase the total of plasma samples to 20 for each group. Plasma was diluted to a 1 to 10 concentration and assayed in duplicates according to the manufacturer's instructions. Intra-assay and interassay coefficients of variation were <8 and <10%, respectively.

Tissue ELISA was carried out on homogenised VAT tissue. Adipose tissue was homogenised in RIPA buffer with added protease inhibitors (Thermo Fisher, UK). Tissue aliquots were added to 1.5 mL PowerBead tubes with 1.4 mm ceramic beads (Qiagen cat no 13113) and shaken at 5000 r.p.m. for 20 s with 10 s rest on ice three times using the MagNALyser instrument (Roche, UK). The homogenate was centrifuged at 5000 r.p.m. for 15 min at 4°C, and the aqueous phase beneath the lipid cake and above the cell pellet was removed to a separate tube. Aliquots were diluted 1:20 in PBS and protein content quantified using the Qubit 4 Protein Assay (Thermo Fisher). The same ELISA kit was used for VAT and plasma (Amsbio EH0564).

Statistical analysis

The microarray data were analysed using R/Bioconductor using an updated chip definition file from the Brainarray resource[22] based on Ensembl gene definitions (ENSG version 22). Raw microarray data were normalized and filtered using the SCAN-UPC method.[23] Expression intensity and UPC filters were set at 0 and 0.5 respectively. Use of these filters suggested 49% of the genes with probes in the microarray chip definition file were not expressed in adipose tissue. This is in line with our previous experience using Affymterix GeneChip microarrays. Filtering also improved the clustering of samples by principle components analysis (*Figure* 1). Changes in differential expression were examined using empirical Bayes and a moderated *t* statistic implemented in the limma package[24] with subject identifier as a blocking factor in linear modelling to account for the repeated measures structure (see section Additive Models and Blocking in the limma manual). Statistical significance was taken as a false discovery rate <5%. Category enrichment analysis was carried out using the camera algorithm[25] and the Broad Institute curated Hallmark Genesets collection.[26] A false discovery rate <5% was taken to indicate significant enrichment of a category.

The qPCR data were analysed using the R/Bioconductor NormqPCR package.[20] The expression data was modelled with a mixed effects linear model using the lme4 R package[27] with group, fat depot, and the interaction between these as fixed factors and subject identifier as a random factor to account for pairing. Post hoc testing of interaction or main effects was carried out using the emmeans[28] package.

Itln1 concentrations were calculated from raw ELISA data in R using the nCal package.[29] After subtraction of blank values as recommended by the kit manufacturer a 5-PL model was fit to the data and concentrations back calculated. Statistical analysis was carried out on log transformed data. Tissue ELISA data were analysed using a linear model with log Itln1 concentration modelled by group. For the plasma ELISA data, the log Itln1 concentrations from Assays 1 and 2 were combined and modelled with a mixed effects model using the lme4 package[27] with assay as a random factor. Post hoc testing was carried out using the emmeans package.[28] Statistical significance was set at $P \leq 0.05$.

Results

Patient characteristics

Twenty-four patients were included in the study with eight subjects in each of the control, CWS, and CC groups. Patient demographics and anthropometric details are shown in *Table* 1. Median weight loss was significantly higher (12.5%, interquartile ratio 6.87–15.5, $P < 0.001$), and SMI was significantly lower in the CC group ($P = 0.002$). Control patients were younger (median 51 years compared with 60 in the CWS group and 70 in the CC group, $P = 0.002$). Final pathology results for all patients can be seen in the Supporting Information.

Microarray analysis

Filtering the microarray data as recommended by the SCAN-UPC authors[23] led to the identification of 8679 probesets (approximately 49%) as unlikely to be expressed in adipose tissue. These were removed from the data set prior to further analysis to improve statistical power. Principle components analysis indicated a substantive difference in VAT vs. SAT transcriptome profiles in the presence of cancer

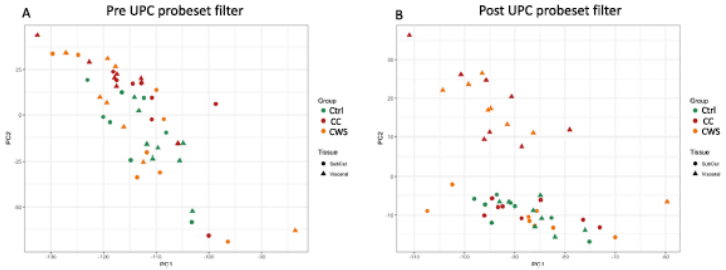

Figure 1 Scatterplots showing separation of adipose depot and group samples by principle components analysis. Before (A) and after (B) removal of non/low expressed genes using the Universal exPression Code (UPC) (Piccolo, 2013) generated filter. CC, cancer cachexia; Ctrl, healthy control; CWS, cancer weight stable.

Table 1 Demographic and selected clinical data for study subjects

Group (n = 8)	Control	Cachexia	Weight stable	P value
Male:Female	4:4	5:3	6:2	/
Age (years)	51 (48–55)	70 (64–77)	60 (57–64)	0.002
BMI	25.5 (23.75–28.25)	24 (20.25–29.25)	31.5 (23–40)	0.227
%weight loss	0	12.5 (6.87–15.5)	0	<0.001
SMI	48.30 (44.32–53.88)	39.99 (38.24–41.13)	55.52 (48.40–64.22)	0.002
VATI	20.97 (11.05–43.06)	23.3 (5.82–38.76)	55.52 (48.40–64.22)	0.196
SATI	50.32 (29.46–63.43)	50.75 (20.15–98.77)	70.35 (34.04–120.67)	0.605
OACC:OSCC:GACC	N/A	6:0:2	6:1:1	

Median (Interquartile range) values are presented.
BMI, body mass index; SMI, skeletal muscle index; VATI, Visceral adipose index; SATI, Subcutaneous adipose index

(*Figure* 1). We first examined mRNA expression differences between the SAT and VAT depots within each group. In the CC group, we found 2101 probesets significantly regulated between the fat depots with fold changes ranging from eight-fold up-regulated to two-fold down-regulated. In the CWS group, we found 1722 probesets significantly regulated with fold changes ranging from nine-fold up-regulated to 2.5-fold down-regulated. In the control group, we found 1659 probesets differentially regulated with fold changes from 4.6-fold up-regulated to 2.3-fold down-regulated. These data indicate that SAT and VAT depots have substantial mRNA expression differences. In addition, the number of differentially expressed probesets is higher in the cancer groups and highest in the CC group (table 2). Comparing VAT between the groups, we found 2200 probesets regulated (10-fold up to 2.3-fold down) between CC and CWS, and 1253 probesets regulated (nine-fold up to 2.3-fold down) between CC and control. No probesets met the statistical cut-off for significance in VAT between the CC and CWS groups. ITLN1, the gene which encodes for the novel adipocytokine Itln1, was the most up-regulated gene with a 10-fold increase in CC vs. control and a nine-fold increase in CWS vs. control (*Figure* 2). Intelectin-2 was also significantly increased in these comparisons. SAT demonstrated no significant differences in any comparison between CC, CWS, and control. Full details of regulated mRNA are available in the Supporting Information. These data demonstrate that whilst the transcriptional profile of SAT is not sensitive to the presence of cancer or CC in our cohort, there are substantial differences in VAT mRNA expression in the presence of cancer.

Table 2 Number of genes regulated in the microarray data with FDR < 5% in each within and between group comparison

	No. regulated genes (FDR < 5%)
VAT vs. SAT in group comparison	
CC VAT vs. SAT	2101
CWS VAT vs. SAT	1722
Control VAT vs. SAT	1659
VAT between group comparisons	
CC vs. control	2200
CWS vs. control	1253
CC vs. cWS	0[a]
SAT between group comparisons	
CC vs. Control	0[b]
CWS vs. Control	0[c]
CC vs. CWS	0[d]

CC, cancer cachexia; CWS, cancer weight stable; FDR, false discovery rate; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue.
[a]lowest adjusted *P* value = 0.7
[b]lowest adjusted *P* value = 0.1
[c]lowest adjusted *P* value = 1
[d]lowest adjusted *P* value = 0.5.

Geneset enrichment analysis

We used the Broad Institute Hallmark-curated genesets collection (17) to examine the data for geneset enrichment. These data are summarised in *Table* 3 and presented in full in the Supporting Information. We first assessed differences between fat depots in each group (*Table* 3). Only Adipogenesis and Fatty Acid metabolism were significantly enriched in all interdepot (VAT compared with SAT) comparisons. In cancer, these genesets were down-regulated in VAT compared with SAT whilst in the control group the opposite pattern was observed, and they were up-regulated in VAT compared with SAT (*Table* 3). The Inflammatory response category was increased in expression in both CC and control VAT compared with SAT, but different genes drove the enrichment signal in each group (*Figure* 3). These data suggest a down-regulation in genes related to growth of adipose tissue in cancer irrespective of weight loss whilst (compared with SAT) inflammation is increased in the VAT of CC patients. We next examined category enrichment in VAT between the groups (Supporting Information). In both CC and CWS VAT, the Adipogenesis and Xenobiotic metabolism (genes encoding proteins involved in the processing of drugs) genesets were decreased in expression compared with control VAT (*Table* 3). Oxidative phosphorylation was significantly down-regulated in the VAT of CC patients compared with that of controls but was not enriched in the comparison of CWS vs. control VAT (*Table* 3).

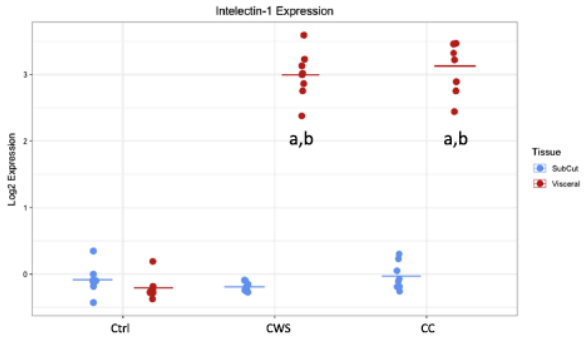

Figure 2 Intelectin-1 mRNA expression values (log2 transformed) from the microarray data. CC, cancer cachexia; Ctrl, healthy controls; CWS, cancer weight stable. a = significantly different from paired subcutaneous values; b = significantly different from control visceral values.

Table 3 Summary of geneset enrichment analysis

Comparison	Geneset	Direction	FDR
Cachexia VAT vs. SAT	Adipogenesis	Down	2×10^{-9}
	Fatty Acid Metabolism	Down	0.004
Cancer VAT vs. SAT	Adipogenesis	Down	1×10^{-6}
	Fatty Acid Metabolism	Down	0.043
Control VAT vs. SAT	Adipogenesis	Up	1.1×10^{-6}
	Fatty Acid Metabolism	Up	4×10^{-8}
Cachexia VAT vs. control VAT	Oxidative Phosphorylation	Down	1.24×10^{-16}
	Adipogenesis	Down	1.24×10^{-16}
	Fatty Acid Metabolism	Down	3.33×10^{-9}
	Xenobiotic Metabolism	Down	0.004
Cancer VAT vs. control VAT	Adipogenesis	Down	0.038
	Xenobiotic Metabolism	Down	0.046
Cachexia VAT vs. cancer VAT	Adipogenesis	Down	0.038
	Xenobiotic Metabolism	Down	0.046

FDR, false discovery rate; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue.

Given our observation of a down-regulated energy metabolism signature in the VAT of cancer patients, we specifically examined the Gene Ontology category Brown Fat Cell Differentiation (GO:0045444) and found this down-regulated in VAT in CC ($P = 5.4 \times 10^{-6}$) and in CWS ($P = 2 \times 10^{-7}$) compared with control VAT. These data suggest that there is a down-regulation of mRNA related to adipose expansion and energy use/generation in the VAT of cancer patients and that there is a stronger signature in cachectic cancer patients. We found no evidence of brown fat cell differentiation in this cohort of cachectic cancer patients.

Quantitative polymerase chain reaction gene validation

We used qPCR to examine selected genes including those detected as regulated in the microarray data and the biological processes of fat browning, inflammation, and adipogenesis.

Microarray candidates

There was a significant depot by group interaction effect for ITLN1 ($P = 0.0006$), the gene that encodes for Itln1. ITLN1 was significantly increased in the VAT of the cancer groups (CC $P = 0.0001$, CWS $P = 0.02$) compared with VAT in the control group but was not different between the cancer groups ($P = 0.5$). ITLN1 expression was also significantly greater in VAT compared with SAT in the cancer groups (CC $P = 0.0004$, CWS $P = 0.03$) with no difference in the control group ($P = 0.9$). There were no significant differences in ITLN1 in SAT in any comparison. ALOX15 (a gene that encodes enzymes which act on various polyunsaturated fatty acid substrates) demonstrated a significant depot by group interaction effect ($P = 0.005$) with increased expression in VAT in the cancer groups compared with control (CC $P = 0.018$ and CWS $P = 0.025$, respectively). ALOX15 was increased in VAT compared with SAT in the cancer groups (weight stable $P = 0.04$, cachectic $P = 0.0046$) but not in the control group ($P = 0.9$). These results are in agreement with the microarray results. PPARA2 also demonstrated a depot by group interaction ($P = 0.004$). However multiple testing was unable to detect specific differences. Inspection of the qPCR data suggests lower expression in the VAT of the cancer groups. The direction of change in the qPCR data for the cancer groups reflected the microarray data. PPARA2 expression between the VAT and SAT depot in the control group was significantly different ($P = 0.03$).

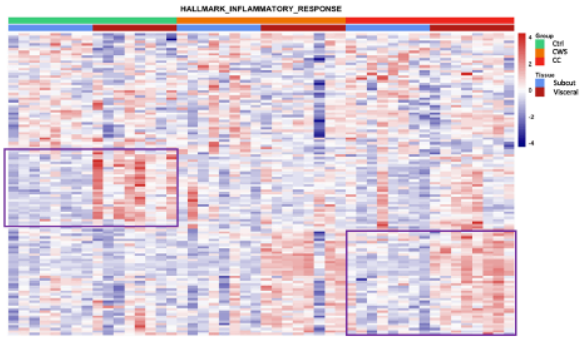

Figure 3 Heatmap of genes in Hallmark Inflammatory Response geneset in subcutaneous and visceral adipose tissue of healthy control (Ctrl), cancer weight stable (CWS), and cachectic cancer (CC) patients. The purple boxes delineate inflammatory genes differentially contributing to geneset enrichment in cancer cachexia and controls. The cancer weight stable group demonstrate a similar signal to the cancer cachexia group but the geneset as a whole did not meet statistical significance for enrichment in VAT versus SAT.

Genes involved in fat browning

There were no differences in UCP1 expression in terms of either a depot by group interaction or main effects in agreement with the microarray results. PPARGC1A demonstrated a main effect of depot (P = 0.003) with VAT showing higher expression than SAT. Post hoc testing revealed that PPARGC1A was significantly up-regulated in the VAT compared with SAT in the control group only (P = 0.01). The direction of change for PPARGC1A in other comparisons was the same as seen in the microarray data but not statistically significant after multiple testing correction. There were no significant differences in PR/SET domain 16 expression in any comparison.

Inflammation

There were no significant differences in ACVR2A1 expression in terms of interactions or main effects, but the direction of changes seen in the qPCR data agreed with the microarray data. There were no significant differences in IL6 expression in terms of interactions or main effects. IL6 was significantly down-regulated in CC compared with control VAT in the microarray data. The direction of change was the same in the qPCR data driven by few patients (*Figure* 4) and not statistically significant. IL18 demonstrated a significant depot by group interaction effect (P = 0.0009) with increased expression in the VAT of both cancer groups compared with control (CWS P = 0.02, CC P = 0.0001). Expression of IL18 was also increased in the VAT compared with the SAT in the cancer groups (CWS P = 0.03, CC P = 0.0006) but not in the control group (P = 0.9).

Adipogenesis

LEPR demonstrated a significant depot by group interaction effect (P = 0.03) with post hoc testing revealing decreased expression in the VAT in the CC compared with control group (P = 0.04). LEPR was also increased in control VAT compared with SAT (P = 0.02). STAT5A did not demonstrate any significant interaction or main effects although the effect of fat depot was borderline significant (P = 0.06) with specific differences undetectable by post hoc testing. Programmed cell death 4 did not demonstrate any significant interaction or main effects.

ELISA results

VAT Itln1 concentrations are shown in *Figure* 5. Linear modelling of log Itln1 concentration by group showed a significant effect of the group (P = 0.04). There was a significant difference between the CC and control groups (mean Itln1 concentrations 43.6 ng/mL and 20.5 ng/mL respectively, P = 0.014) but no statistically significant difference between CWS (mean Itln1 concentration 30.9 ng/mL) and either of the other two groups.

Given that Itln1 was raised in the VAT of CC patients, we wanted to investigate if it was also raised in the circulating fraction of these patients, and so, plasma ELISA was undertaken. Patients demographics of those included in the

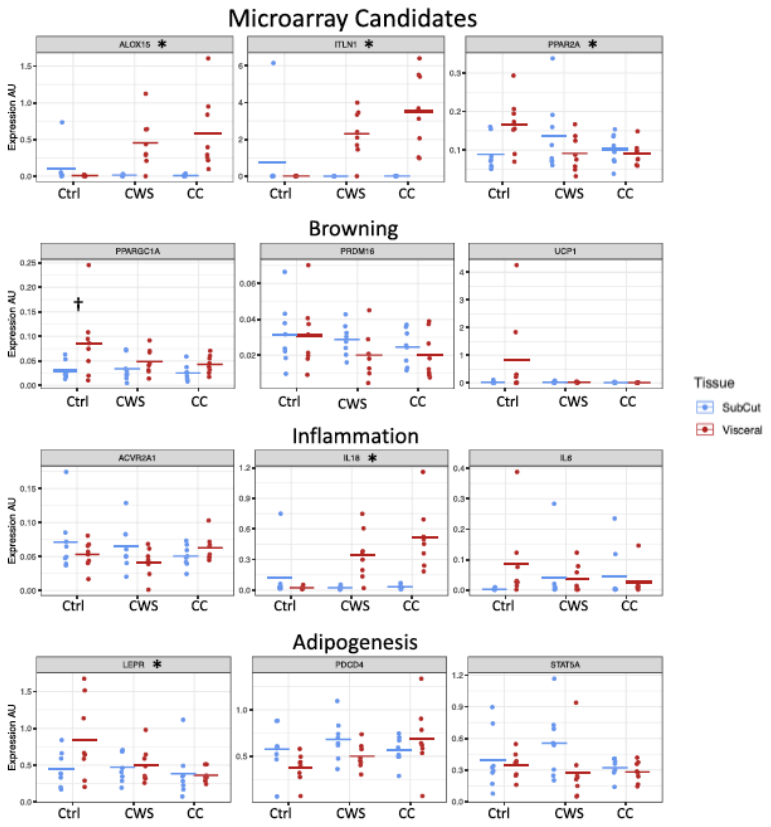

Figure 4 Data from qPCR of selected genes. Categories of genes validated by qPCR were significantly different in microarray data (ALOX15, ITLN1, and PPARA2), involved in browning (PPARGC1A, PRDM16, and UCP1), inflammation related (ACVR2A1, IL6, and IL18), involved in adipogenesis (LEPR, STATSA, and PDCD4). * = significant group × depot interaction; † = no significant interaction but significant difference between same depot in control group. See main text for full reporting of statistically significant differences. AU, arbitrary units; CC, cancer cachexia; Ctrl, healthy controls; CWS, cancer weight stable.

plasma ELISA study are summarised in *Table* 4a,b4b. Plasma Itln1 concentrations are shown in *Figure* 6. Mixed effects linear modelling with group as a fixed effect and assay run as a random effect was used to model log Itln1 concentrations. There was a significant effect of group (P = 0.028). Post hoc testing using Tukey's correction demonstrated a significant difference in plasma Itln1 concentrations between the CC and CWS groups (mean Itln1 concentrations 2.6 ng/mL and 1.9 ng/mL respectively, P = 0.02). There was no significant difference between the control group (mean Itln1 concentration 2.3 ng/mL) and either of the other groups.

Discussion

Different mechanisms have been previously proposed to account for the changes in adipose tissue seen in cachexia. In

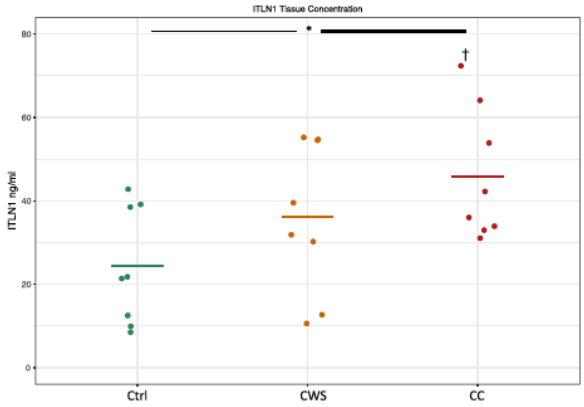

Figure 5 Intelectin-1 visceral adipose tissue protein concentration (nanogram per millilitre) as measured by enzyme-linked immunosorbent assay across the control (Ctrl), cancer weight stable (CWS) and cancer cachexia (CC) groups. * = significant effect of group; † = significantly different from control group.

Table 4a Demographic details of total patients included in the ITLN-1 plasma enzyme-linked immunosorbent assay (ELISA). This cohort includes patients recruited for both array and ELISA studies

Group (n = 20 per group)	Control	Cachexia	Weight stable	P value
Male:Female	14:6	14:6	14:6	/
Age (years)	49 (11)	69 (10)	64 (9)	<0.001
BMI	25.66 (2.81)	25.06 (4.85)	29.93 (7.79)	0.187
%weight loss	0	11.60 (6.58)	0.26 (0.71)	<0.001
SMI	50.69 (7.55)	41.54 (4.82)	55.92 (12.48)	<0.001
VATI	26.23 (20.04)	41.50 (36.65)	111.90 (215.25)	0.008
SATI	52.98 (27.79)	71.85 (59.75)	71.87 (43.75)	0.460

Data are mean (SD).
BMI, body mass index; SATI, subcutaneous adipose index; SMI, skeletal muscle index, VATI, visceral adipose index.

Table 4b Demographic details of additional patients recruited the ITLN-1 plasma enzyme-linked immunosorbent assay. Excludes those patients recruited to the microarray study

Group (*n* = 12)	Control	Cachexia	Weight stable	*P* value
Male:Female	10:2	9:3	8:4	/
Age (years)	48 (14)	69 (10)	68 (10)	<0.001
BMI	25.77 (2.67)	25.35 (3.68)	29.05 (7.61)	0.697
%weight loss	0	11.21 (6.78)	0.43 (0.90)	<0.001
SMI	51.42 (7.97)	42.56 (4.84)	56.04 (14.74)	0.019
VATI	23.65 (15.11)	51.41 (40.68)	151.78 (279.69)	0.016
SATI	51.24 (19.89)	74.69 (60.69)	69.77 (45.06)	0.620

BMI, body mass index; SATI, subcutaneous adipose index; SMI, skeletal muscle index, VATI, visceral adipose index.

CC, human and animal studies have shown increased rates of lipid mobilisation and loss of adipose tissue before any demonstrable changes in loss of muscle mass.[30] An increased rate of adipose tissue loss has also been associated with worsening prognosis in cancer patients highlighting the importance of gaining a greater understanding of the mechanisms involved. To the best of our knowledge, this is the first study to examine both SAT and VAT depots in CC

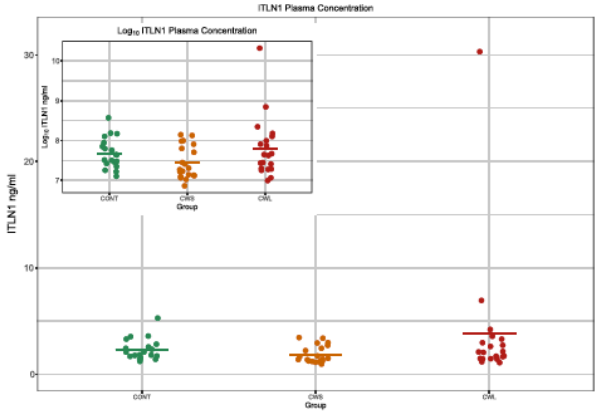

Figure 6 Intelectin-1 plasma protein concentration (ng/ml) as measured by enzyme-linked immunosorbent assay across the control (Ctrl), cancer weight stable (CWS) and cancer cachexia (CC) groups. * = significant effect of group; ‡ = significantly different from Ca group.

patients. We have shown that SAT and VAT have unique gene expression signatures, and VAT has an altered gene expression signature in cancer. We found increased expression of Itln1 and Intelectin-2 in particular suggesting these adipokines may play a role in adipose changes in response to cancer. Itln1 may be a target for therapeutic manipulation.

The gene which showed the largest difference in expression was ITLN1; a 34 kDa novel adipokine. Expression of ITLN2 was also high but not as great as ITLN1 and so ITLN1 formed the focus of our study. ITLN1 is preferentially produced by stromovascular cells in VAT[31] but has also been identified in human epicardial fat cells, mesothelial cells, airway goblet cells, and cells lining the gut and ovaries.[32] A series of studies have linked raised levels of ITLN1 to various cancers.[33–40] ITLN1 has been suggested to promote cancer cell growth by triggering genomic instability via phosphatidylinositol-3 kinase downstream effector signalling pathways.[41] The role of ITLN1 in CC however, has not been well characterised. Randomized control trials have shown that weight loss significantly increases plasma ITLN1 concentration[42] whereas hyper-insulinaemic induction in healthy individuals reduces ITLN1 plasma concentration.[43] Decreased levels of ITLN1 have been associated with obesity in patients with ovarian cancer,[43] and levels of ITLN1 measured before patients were diagnosed with colorectal cancer have been confined to nonobese individuals suggesting it has a role in weight loss.[44]

Our microarray results confirmed that expression of ITLN1 was higher in VAT in cancer although not confined to CC patients. Plasma levels of Itln1 were significantly increased in CC compared with CWS groups but showed no difference with controls suggesting it possibly has a role in cancer induced weight loss. However, as in the previous studies, we found that plasma Itln1 differences were driven by a few individuals with high levels. High interindividual variability in plasma ITLN1 likely limited the ability to detect a statistically significant effect across all patient groups. Larger patient studies will be required in the future to ascertain any true difference in circulating ITLN1 in cancer. The significant increase in intelectin seen in the VAT ELISA of cachectic patients suggests a possible role in the pathogenesis of cachexia. There was a clear increasing gradient from controls through CWS to CC in VAT Itln1 level. This was not reflected in the plasma Itln1 protein levels. These results are similar to those we have previously seen with zinc a-glycoprotein [ZAG][6] in CC. This pattern of increased tissue protein levels but no change in circulating levels could be explained by cells outside adipose tissue contributing to circulating protein. Alternatively, there could be a predominantly paracrine role for adipose tissue secreted protein that is not released into the circulation.

We found no evidence of increased browning in white adipose tissue in our cohort. Previously increased thermogenic activity of brown and beige adipose tissue has been shown to contribute to the increased energy expenditure and weight loss in rodent models of cachexia.[45–47] White adipose tissue browning is associated with increased expression of UCP1, which uncouples mitochondrial respiration towards thermogenesis instead of adenosine triphosphate synthesis, leading to increased lipid mobilization and energy expenditure.[48] Whilst browning of white fat depots contributes to futile energy cycling in cachexia in animal models the importance of this is uncertain.[49,50] Genes involved in fat browning in the present study were

not up-regulated suggesting browning has a limited role in the pathogenesis of cachexia in the recruited patients and highlights the importance of patient-based research. These data provide the basis for future studies that further examine the role of adipose biology in the metabolic effects of cancer in humans.

Cachexia is thought to be a chronic inflammatory state, and changes in inflammatory mediators in adipocytes may be capable of inducing changes in adipose tissue homeostasis.[51] Several studies have shown adipose tissue to be the target of several pro-cachectic factors as well as adipose tissue itself being an important source of inflammatory mediators.[52–56] The majority of studies assessing inflammation in cachexia have examined inflammatory markers from a systemic point of view rather than a tissue-specific one with IL6 in particular frequently found to be raised in the plasma of CC patients.[57] IL6 was down-regulated in adipose tissue in CC suggesting a possible homeostatic response. Specific adipose tissue inflammation is important to consider as it may be an event very early on in the cachectic process prior to any systemic effects being demonstrated.

Adipogenesis is the process by which preadipocytes differentiate into mature adipocytes able to store triglycerides and secrete adipokines. Some studies have shown that adipogenic genes are down-regulated in animal epididymal and retroperitoneal fat in cachexia.[54,58] Co-culture studies have demonstrated inhibition of pre-adipocyte maturation in the presence of tumour cells that was associated with increased inflammatory cytokines.[59] Changes in adipogenesis are likely to precede the clinical signs of fat wasting and tissue inflammation. It is possible therefore that factors such as increased infiltration of macrophages and production of inflammatory cytokines silence adipogenic genes and/or increase lipolysis leading to adipose wasting in cachexia.

The current study confers similar limitations to other microarray studies most notably in the small sample size. However exploratory work of this nature is valuable as it provides testable hypotheses to be taken forward. Another limitation of this study was the availability of omental and perinephric adipose tissue in the cancer and control groups, respectively. Differences in these two depots are not well defined. In rodents, there are possible depot-specific differences in innervation, but this has not been documented in humans.[60] A very small study in humans has suggested blood flow in omental fat may be higher than in perinephric fat though the difference was not significant.[61] This may potentially the affect removal of lipolytic products and the rates of lipolysis. The patients studied were predominantly male, and we are therefore unable to draw conclusions about sex-specific differences. There was no longitudinal follow-up of patients done, and so, it is unclear whether the patients in the CWS group went on to develop cachexia and therefore may have been pre-cachectic at the time of biopsy. We also excluded patients in the control and CWS groups who were sarcopenic on CT scan. In doing so, we have assumed that a reduction in SMI in the cachectic group was because of disease. Given that these patients have a median age of 70, it is possible that some were sarcopenic prior to the start of the disease process. We chose to define patients based on this specific definition of cancer cachexia (>2% weight loss and low muscularity) as it is the previous definition that has been associated with histological wasting.[16] Notably, all but one of the participants in our study defined as having cancer cachexia had >5% weight loss (over 6 months). Reanalysis of the ITLN1 qPCR data after removal of this single subject did not affect the conclusions of that analysis.

Conclusions

This exploratory analysis confirms differential gene expression in adipose depots in patients with cancer with and without cachexia and highlights the importance of VAT in cancer. VAT may have a fundamental role in cachexia, but the down-regulation of energy metabolism genes implies a limited role for browning in cachectic patients in our cohort as suggested in pre-clinical models. Future mechanistic studies are important to evaluate the effects of VAT in cachexia and in particular the role of Itln1.

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Ethical standards

The study conforms to the declaration of Helsinki. All patients provided written, informed consent. The manuscript complies with the ethical guidelines for authorship and publishing in the *Journal of Cachexia, Sarcopenia and Muscle*.[62]

Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1. Supporting Information

Conflict of interest

The authors declare no conflicts of interest.

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cancers

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Article

Plasma Metabolomics Identifies Lipid and Amino Acid Markers of Weight Loss in Patients with Upper Gastrointestinal Cancer

Janice Miller [1], Ahmed Alshehri [2], Michael I. Ramage [1], Nathan A. Stephens [2], Alexander B. Mullen [3], Marie Boyd [3], James A. Ross [1], Stephen J. Wigmore [1], David G. Watson [3] and Richard J.E. Skipworth [1,*]

1 Clinical Surgery, University of Edinburgh, Royal Infirmary of Edinburgh, Edinburgh EH16 4SA, UK; j.miller@ed.ac.uk (J.M.); mramage@exseed.ed.ac.uk (M.I.R.); j.a.ross@ed.ac.uk (J.A.R.); s.wigmore@ed.ac.uk (S.J.W.)
2 The Royal Liverpool University Hospital, Prescot Street, Liverpool L7 8XP, UK; Ahmed.Alshehri@strath.ac.uk (A.A.); nathan.stephens@liverpoolft.nhs.uk (N.A.S.)
3 Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow G1 1XQ, UK; a.mullen@strath.ac.uk (A.B.M.); marie.boyd@strath.ac.uk (M.B.); d.g.watson@strath.ac.uk (D.G.W.)
* Correspondence: Richard.Skipworth@nhslothian.scot.nhs.uk; Tel.: +44-(0)131-242-3615

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Abstract: Cachexia is a multifactorial wasting syndrome associated with high morbidity and mortality in patients with cancer. Diagnosis can be difficult and, in the clinical situation, usually relies upon reported weight loss. The 'omics' technologies allow us the opportunity to study the end points of many biological processes. Among these, blood-based metabolomics is a promising method to investigate the pathophysiology of human cancer cachexia and identify candidate biomarkers. In this study, we performed liquid chromatography mass spectrometry (LC/MS)-based metabolomics to investigate the metabolic profile of cancer-associated weight loss. Non-selected patients undergoing surgery with curative intent for upper gastrointestinal cancer were recruited. Fasting plasma samples were taken at induction of anaesthesia. LC/MS analysis showed that 6 metabolites were highly discriminative of weight loss. Specifically, a combination profile of LysoPC 18.2, L-Proline, Hexadecanoic acid, Octadecanoic acid, Phenylalanine and LysoPC 16:1 showed close correlation for eight weight-losing samples (≥5% weight loss) and nine weight-stable samples (<5%weight loss) between predicted and actual weight change ($r = 0.976$, $p = 0.0014$). Overall, 40 metabolites were associated with ≥5% weight loss. This study provides biological validation of the consensus definition of cancer cachexia (Fearon et al.) and provides feasible candidate markers for further investigation in early diagnosis and the assessment of therapeutic intervention.

Keywords: cachexia; cancer; metabolomics; high resolution mass spectrometry

1. Introduction

Cancer cachexia has been defined as "a multifactorial syndrome characterized by an ongoing loss of skeletal muscle mass that cannot be fully reversed by conventional nutritional support and leads to progressive functional impairment" [1]. The agreed consensus diagnostic criterion for cachexia is either weight loss >5% over 6 months or weight loss >2% in individuals already showing signs of depletion (BMI < 20 kg/m^2 or skeletal muscle index consistent with sarcopenia) [1]. Cancer cachexia is characterized by loss of adipose tissue, skeletal muscle, and appetite, and impacts negatively the quality of life of patients with cancer, response to treatment and survival [2]. Therefore, managing cachexia should be considered a central component of cancer patient treatment.

As cachexia develops progressively through various stages, from pre-cachexia to cachexia to refractory cachexia, the identification of biomarkers that relate to stage and diagnosis would be particularly important to prevent or delay deterioration [1]. Potential markers of cachexia may also have value through future studies as outcome measures of therapeutic intervention. Imaging methods such as CT and MRI are currently considered precise measures of body composition but have several limitations, including cost, availability, and exposure to radiation (in the case of CT) [3].

Recent progress in high-throughput analytical technologies and bioinformatics now permits simultaneous analysis of hundreds of compounds constituting the metabolome [4]. Metabolomic analyses give complex fingerprints that appear to be characteristic of a given metabolic phenotype or diet. Several previous studies have attempted to quantify urinary and plasma metabolites associated with cachexia. They have identified metabolites that are possibly discriminative of cachexia, indicating that there may be scope for clinical application of metabolomic biomarkers of cachexia [5–9].

We have previously analysed urinary proteomics as a measure of degradation products in the circulating fraction, allowing us to discriminate between weight-losing and weight stable cancer patients [10]. Building upon the theory that metabolites produced from tissue breakdown are likely to be found in plasma, we investigated whether we could use liquid chromatography mass spectrometry (LC/MS)-metabolomics to detect plasma metabolites associated with weight loss in upper GI cancer patients. In particular, we aimed to identify a metabolic signature that relates directly to patient weight loss. Plasma was selected as the biofluid of choice as it has been shown previously that several end products of muscle catabolism (e.g., creatinine and methylhistidine) can be easily measured within [11].

2. Results

Plasma samples were analysed from upper GI cancer patients (n = 18) taken from a cross-sectional cohort of upper GI cancer patients who were recruited to two previously-published studies of muscle transcriptomics (n = 65 pre-surgical rectus abdominis biopsies, and n = 12 pre- and post-surgical resection muscle biopsies) [12,13]. In these previous studies, quantitative significance analysis of microarrays produced an 83-gene signature that was able to identify patients with >5% weight loss, while this molecular profile was unrelated to markers of systemic inflammation. Comparison with healthy control muscle revealed that despite differences in the muscle transcriptome at baseline (941 genes regulated), the muscle of patients at post-surgical resection follow-up was similar to control muscle (two genes regulated). Baseline, pre-surgical plasma samples were available for 18 of these patients. Therefore, for the present study, there were nine patients who had experienced ≥5% weight loss in the previous 6 months (≥5% WL group), and nine patients with <5% weight loss (weight stable group: WS). Cancer cachexia had been primarily defined as ≥5% weight loss in order to identify patients who would have experienced dynamic wasting and thus be more likely to have identifiable markers and metabolic signatures. If the patients were analysed as two separate groups according to these weight loss criteria, the mean percentage weight loss in the ≥5% WL group was 14.39% compared with 2.13% in the WS group (p = 0.001). There were more males in the WS group. There was no significant difference in age, body mass index (BMI), Skeletal Muscle Index (SMI), Subcutaneous Adipose Tissue Index (SATI) or Visceral Adipose Tissue Index (VATI) between the groups. However, patients in the ≥5% WL group did demonstrate higher CRP levels compared with WS patients. These body composition and systemic inflammatory phenomena are all associated with worsened outcomes in cancer patients [14–16], confirming the ≥5% WL patients as a high-risk group. Two patients in the WS group had >2% weight loss and low SMI [according to Martin criteria [17] on the CT scan. Patients had a mixture of upper gastrointestinal cancers—predominantly those of the oesophagus and pancreas (Table 1). On the overall metabolomic analysis, a total of 40 metabolites were significantly associated with ≥5% weight loss according to univariate analysis using a T test. The metabolites with the highest fold change were L-phenylalanine and various species of LysoPE, LysoPA and LysoPC. These metabolites, as well as free fatty acid levels (FFAs), were all elevated in the ≥5% WL group.

Table 1. Patient details.

Demographics	Group 1 Weight Stable (n = 9)	Group 2 ≥5% Weight Loss (n = 9)	p Values
Male: Female	8:1	5:4	N/A
Age (years)	61 (4.65)	66 (10.53)	0.167
% Weight loss	2.13 (1.35)	14.39 (6.56)	0.001 *
SMI	47.17 (6.26)	45.82 (7.72)	0.536
SATI	46.25 (20.38)	58.43 (33.86)	0.379
VATI	57.57 (55.28)	42.10 (33.48)	0.506
BMI (kg^2/m^2)	24.93 (4.42)	26.29 (4.64)	0.534
CRP (mg/L)	17.88 (27.06)	32.56 (50.44)	0.453
Cancer type	Pancreatic – 1 Oesophageal – 6 Gastric - 2	Pancreatic – 6 Oesophageal – 2 Duodenal - 1	N/A
Disease stage	1 (n = 1) 2 (n = 0) 3 (n = 6) 4 (n = 1) Unknown (n = 1)	1 (n = 1) 2 (n = 5) 3 (n = 1) 4 (n = 2) -	
Pre-operative chemotherapy	4	2	N/A

All data are mean (standard deviation). SMI—Skeletal muscle index, SATI—subcutaneous adipose tissue index, VATI—Visceral adipose tissue index, BMI—Body mass index, CRP = C Reactive protein. * Denotes statistically significant result.

Figure S1 shows the principal components analysis (PCA-X) of all 18 samples. Three quality control (QC) samples were also included in the run and indicated that the instrument stability was good for the duration of the run. PCA-X, an unsupervised model in SIMCA-P, produces a natural scatter of the samples based on their characteristic metabolomics footprints. In general, there was no separation of samples according to the cachexia threshold defined as ≥ 5% weight loss (Figure S1).

Supervised models enable identification of metabolites that have the most significant contribution to a given clustering pattern. In SIMCA, supervised analysis can be carried out using orthogonal partial least squares discriminant analysis (OPLS-DA) or orthogonal partial least squares (OPLS) models. An OPLSDA model was not very successful in classifying the samples without the need to omit several. However, an OPLS model based on six metabolites (Table 2) was successfully produced for all but one of the patients who had ≥5% weight loss. This individual patient sample was omitted from the OPLS model since it clustered with the WS samples. The model plotted predicted against actual weight loss, and had a CVANOVA of 0.0014. The correlation line had an r value of 0.976 when fitted through the samples (Figure 1). Therefore, these six metabolites provide a useful metabolomic signature for further longitudinal testing.

Table 3 shows the metabolites that were found to be significantly different between the two weight loss categories. The ratio represents the intensity of the metabolites relative to the WS patient group.

Table 2. The six metabolites used to produce the OPLS model shown in Figure 1

m/z	Rt Min.	Metabolite	VIP Value
520.339	4.4	Lyso-PC 18:2	1.82
116.071	13.0	L-Proline	1.43
255.233	4.3	Hexadecanoic acid	0.54
281.249	3.8	Octadecenoic acid	0.42
166.086	10.0	Phenylalanine	0.36
480.344	4.4	Lyso-PC 16:1	0.20

M/Z—Mass to charge ratio, Rt—Retention time, VIP—Variable importance in projection.

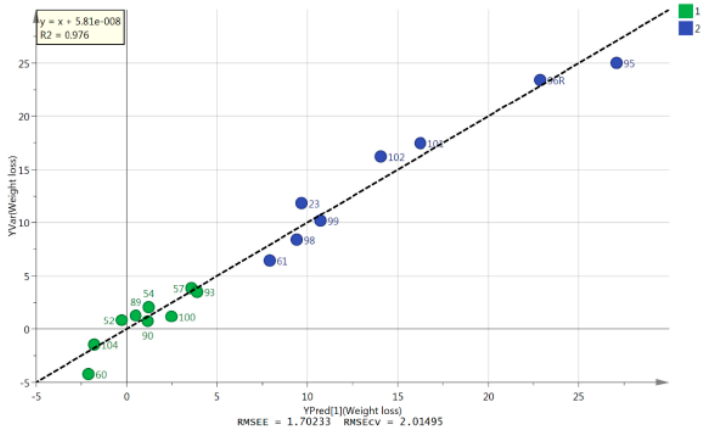

Figure 1. Orthogonal partial least squares (OPLS) model. OPLS model showing close correlation for eight ≥5% WL samples and nine WS samples between predicted and actual weight change (CVANOVA = 0.0014) based on six variables listed in Table 2. Green circles (group 1) = WS, blue circles (group 2) = ≥5% WL.

Table 3. Significant metabolites that differ between ≥ 5% WL and WS groups (n = 9 and 9 respectively).

Polarity	m/z	Rt(min)	Metabolite	*p* Value	Ratio WL/WS
			Amino acids		
P	116.071	13.0	L-Proline	0.015	1.36
P	166.086	10.0	L-Phenylalanine	0.619	0.87
			Fatty acids		
N	214.048	4.3	sn-glycero-3-Phosphoethanolamine	0.006	1.78
N	255.233	4.3	Hexadecanoic acid	0.049	1.21
N	277.217	3.9	Octadecatrienoic acid	0.010	1.60
N	279.233	4.2	Linoleate	0.002	1.36
N	281.249	3.8	Octadecenoic acid	0.023	1.22
N	293.249	4.2	Nonadecadienoic acid	0.019	1.24
N	303.233	4.1	Eicosatetraenoic acid	0.022	1.37
N	305.249	4.2	Eicosatrienoic acid	0.054	1.50
N	327.233	4.2	Docosahexaenoic acid	0.025	0.81
N	329.249	4.1	Docosapentaenoic acid	0.009	1.46
N	331.264	3.9	Docosatetraenoic acid	0.014	1.68
P/N	380.255	5.1	Sphingenine phosphate	0.033	1.28
			Lipids		
N	214.048	4.3	Glycerophosphoethanolamine	0.006	1.78
N	381.205	4.6	LPA 14:0	0.010	1.73
N	393.241	4.4	LPA 16:0 ether	0.048	1.37
N	433.236	4.4	LPA 18:2	0.001	1.67
N	435.252	4.5	LPA18:1	0.006	1.40
N	437.267	4.2	LPA 18:0	0.028	1.23
P/N	454.292	4.6	LPE 16:0	0.040	1.44
N	457.235	4.3	LPA 20:4	0.001	1.62
N	464.278	4.4	LPC 14:1	0.007	1.36
P	468.308	4.6	LPC 14:0	0.026	1.58
P/N	476.278	4.4	LPE 18:2	0.013	1.98
P/N	478.292	4.4	LPE 18:1	0.013	2.02

Table 3. *Cont.*

Polarity	m/z	Rt(min)	Metabolite	*p* Value	Ratio WL/WS
P/N	480.308	4.4	LPE 18:0	0.012	2.07
P/N	480.344	4.4	LPC16:1	0.089	1.32
N	485.267	4.3	LPA 22:4	0.007	1.96
P/N	496.339	4.4	LPC 16:0	0.040	1.34
N	498.262	4.3	LPE 20:5	0.053	2.13
P/N	500.278	4.4	LPE 20:4	0.002	2.36
N	504.31	4.4	LPE 20:2	0.002	1.65
N	514.294	4.3	LPC 18:4	0.005	2.28
P/N	520.339	4.4	LPC 18:2	0.001	1.75
P/N	524.278	4.3	LPE 22:6	0.032	1.45
N	526.294	4.3	LPE 22:5	0.004	2.51
N	528.31	4.3	LPE22:4	0.004	2.01
P/N	544.338	4.3	LPC 20:4	0.014	1.81
P/N	546.354	4.3	LPC 20:3	0.026	1.91
P	570.356	4.2	LPC 22:5	0.009	1.89
P	731.605	4.2	SMd18:0/18:1	0.052	1.29

M/Z = Mass to charge ratio, WS= Weight stable, WL = Weight losing, PE = phosphatidyl ethanolamine, PC = phosphatidyl choline, PA = phosphatidic acid, L = lyso, P = Detection in positive ion mode, N = Detection in negative ion mode.

Figure 2 is a heat map showing the relative abundance of the lyso- lipids in these plasma samples. Lyso-PC18:2 was almost as abundant as Lyso-PC 16:0 and was elevated by 1.75 fold. Beyond these two lyso-lipids, the metabolite abundance was much lower but there were many more minor lipids showing similar or greater fold changes in the ≥5% WL group compared with WS.

purple= > 30%, yellow >1% blue >0.1%

m/z	Rt min			Group 1 (WS)	Group 2 (≥ 5%WL)
496.339	4.4	C24H50NO7P	LysoPC 16:0		
520.339	4.4	C26H50NO7P	LysoPC18:2		
544.338	4.3	C28H50NO7P	LysoPC20:4		
435.252	4.5	C21H41O7P	LysoPA18:1		
464.278	4.4	C22H44NO7P	LysoPC 14:0		
546.354	4.3	C28H52NO7P	LysoPC20:3		
437.267	4.2	C21H43O7P	LysoGP 18:0		
480.344	4.4	C24H50NO6P	LysoPC 16:1		
478.292	4.4	C23H44NO7P	LysoPE18:2		
570.356	4.2	C30H52NO7P	LysoPC22:5		
480.308	4.4	C23H46NO7P	LysoPE18:0		
454.292	4.6	C21H44NO7P	LysoPE 16:0		
526.292	4.3	C27H44NO7P	LysoPE22:5		
457.235	4.3	C23H39O7P	LysoPG 20:4		
524.278	4.3	C27H44NO7P	LysoPC18:0		
508.341	4.3	C25H52NO7P	LysoPE 18:0		
504.31	4.4	C25H48NO7P	LysoPE20:2		
485.267	4.3	C25H43O7P	LysoPA20:4		
363.158	4.4	C16H29O7P	LysoPA 16:0		
514.294	4.3	C26H46NO7P	LysoPC18:4		
528.31	4.3	C27H48NO7P	LysoPE22:4		
540.309	4.4	C28H48NO7P	Lyso PC 20:5		

m/z – mass to charge ratio, Rt – Retention time.

Figure 2. Heat map showing relative levels of lysolipids.

3. Discussion

In this study, we performed LC-MS based metabolomics analysis to reveal the metabolic profile of weight loss in cancer. We were able to demonstrate distinct profiles associated with the presence or absence of ≥5% WL. Most of the metabolites identified within these profiles fell within the lipid pathways. The clearest finding was that several long chain fatty acids and lysolipids were elevated in the plasma of the patients with higher weight loss. Lysolipids are very abundant in plasma [18] and Lyso-PC 16:0 was the most abundant compound by response in this set of samples; an increase of 1.34 fold between ≥5% WL and WS patients represents a major shift in metabolic output.

The OPLS model shown in Figure 1 indicates a strong association between weight loss and six metabolites. Two of the metabolite markers were lysolipids, two were amino acids, and two were fatty acids. There is a risk of overfitting data when the numbers of samples are small. However, in the present study, there was a clear indication that a small number of markers may be used to model the degree of weight loss with accuracy. One patient with ≥5% WL was removed from the analysis as this sample clustered with the WS group, but for this small cohort alone, our six identified metabolites, as a diagnostic test for cachexia (≥5% WL), would be 95% accurate. Validation of these markers will require larger studies, ideally with sequential assessment. Therefore, the current study demonstrates an association between lipolytic activity in the plasma of cancer patients with weight loss. The changes in the amino acids found add credence to the importance of muscle wasting in cancer cachexia and indeed, most current research into cancer cachexia focuses on this area. However, the importance of lipid metabolism is re-emerging as an area of priority [19].

Previously, cachexia in patients with cancer of the oesophagus and pancreas has been linked with high levels of plasma glycerol and free fatty acids [20,21]. Weight-losing cancer patients have been shown to have increased turnover of both glycerol and fatty acids compared with cancer patients without weight loss [22]. Some, however, have suggested that observed increases in lipolysis and triglyceride-fatty acid cycling in cachectic patients with oesophageal cancer are due to alterations in nutritional status rather than disease presence [23]. Cachectic ovarian cancer patients have been shown to have increased levels of free fatty acids, monoacylglycerides and diglycerides in their serum and ascitic fluid [24]. Whilst it is difficult to determine where fatty acid and lipid metabolites originate, both lysolipids and fatty acids are markers of lipolysis [25,26]. Free fatty acids may also provide energy for the tumour, with the glycerol molecules released during the breakdown of triacylglycerides being used for gluconeogenesis by the liver [27,28]

Previous attempts to profile metabolites associated with cachexia have yielded varying results and differences in important metabolites produced in each study. Metabolomics research in cachexia began in 2008 in the C26 mouse model [5]. This was the first study to demonstrate a distinct metabolomics-based profile associated with the onset of muscle wasting and identify increased levels of very low and low density lipoprotein associated with aberrant glycosylation of β-Dystroglycan (β-DG, a marker of muscle wasting in this mouse model) [29]. Human metabolomics studies in cancer cachexia have investigated urine and plasma from weight-losing patients. These studies also found large numbers of glycerophospholipids and metabolites associated with amino acid metabolism and were able to identify occult sarcopenia in patients with cancer [6,30]. Recent studies have attempted to separate pre-cachectic, cachectic and weight stable cancer patients and healthy controls using serum metabolomics, and enabled identification of fifteen highly discriminative metabolites [8]. The present study was able to discriminate using weight-losing from weight-stable patients using only six metabolites. The only study to find a markedly different metabolic pattern to the present study analysed patients using three analytical platforms, namely gas chromatography mass spectrometry, capillary electrophoresis mass spectrometry, and LC/MS. The authors found a significant reduction in amino acids and glycerophospholipids associated with cachexia, a difference that has not previously been described in this condition, plus a high increase in cortisol levels [9]. All bar one of these studies [6] used 5% weight loss as a cut off to stratify patients, in a similar fashion to the present study. Future

studies should investigate the relationship between metabolomics and dynamic assessments of tissue loss over time, e.g., using serial CT body composition analysis.

Blood-based metabolomics is a promising method for cachexia research. However, as seen previously, results are often difficult to replicate due to the heterogeneity of the populations and study sizes. All the patients included in the present study were suitable for potentially curable surgical resection of their cancer and therefore had localized/non-metastatic disease and similar measures of muscle volume, indicating that the identified metabolites might be early markers of fat wasting preceding muscle loss. This hypothesis is supported by the fact that two of the patients in the WS group were "cachectic" according to the consensus definition, by virtue of having ≥2%WL and low SMI on CT, and yet, they did not group with the identified panel of six metabolites. This finding may be explained by a lack of dynamic wasting, and that CT-identified sarcopenia may be the pre-cancer patient norm, rather than a consequence of disease.

One obvious limitation of this study is the small number of samples used, involving differences in sex ratios and cancer types between the groups. This was an exploratory study involving patients without refractory cachexia and was not designed to identify sex- or tumour-specific differences. This study was limited to patients with UGI cancers and therefore, may not be applicable to all cancer types. Previous serum metabolomic studies in pancreatic and oesophageal cancer have confirmed that dysregulation of lipid metabolism is a key component of these conditions, although the exact tissue site of these processes is unknown [31,32]. In the present study, differences in tumour type between patient groups are unlikely to explain the identified six-metabolite signature, due to the close correlation with patient weight loss. We were able to demonstrate distinct metabolic profiles consistent with the consensus cachexia definition of ≥5% weight loss. These findings support our previous muscle transcriptomic studies, and they give further biological validation to the 2011 Fearon consensus definition of cancer cachexia [1] as a valid patient inclusion criterion in clinical trials. Furthermore, these results provide a 6-metabolite profile for further investigation as a marker of cachexia in longitudinal studies, with the opportunity to explore early diagnosis and response to therapeutic intervention.

4. Materials and Methods

4.1. Participants

Patients were all over 18 years of age and were recruited from the regional upper gastrointestinal (GI) multi-disciplinary team meeting. Written informed consent was obtained from all subjects and ethical approval received from Lothian Research Ethics Committee (UK, ethic code: 06/S1103/75) Participating patients had a diagnosis of upper GI cancer (oesophageal, gastric, pancreatic or duodenal) and were undergoing surgery with the intent of curative resection of the primary tumour. All patients had normal kidney function. Clinical details, degree of weight loss from self-reported pre-illness stable weight, and body mass index (BMI) were recorded.

4.2. CT Body Composition Analysis

Skeletal muscle cross-sectional area (CSA) was measured from routine staging CT scans performed prior to any surgical intervention. A transverse CT image from the third lumbar vertebrae (L3) was assessed for each scan date and tissue volumes estimated using semi-automated software. Cross-sectional area for muscle, subcutaneous and visceral adipose tissue was normalized for stature (cm^2/m^2) to calculate the skeletal muscle, subcutaneous and visceral adipose indices (SMI, SATI and VATI respectively).

4.3. Sample Collection and Storage

Fasting venous blood samples were taken at induction of anaesthesia approximately four to six weeks after the cessation of any neoadjuvant chemotherapy. Samples were allowed to clot at room temperature. Serum was separated by centrifugation at 1300 RPM for 12 min at a temperature of

20 degrees Celsius. C-reactive protein (CRP) was measured in Clinical Chemistry, Royal Infirmary, Edinburgh (fully accredited by Clinical Pathology Accreditation Ltd., UK) using standard automated methods. A CRP ≥5 mg/L was considered consistent with the presence of systemic inflammation. Samples were stored locally at −80 °C until transported to the metabolomic facility (Strathclyde Institute of Pharmacy and Biomedical sciences) in cool bags at −30 °C.

4.4. Chemicals and Solvents

HPLC grade Acetonitrile (ACN) was purchased from Fisher Scientific (Loughborough, UK) and HPLC grade water was produced by a Direct-Q3 UltrapureWater System (Millipore, Watford, UK). AnalaR-grade formic acid (98%) was obtained from BDH-Merck (Poole, UK). Authentic stock standard metabolites (Sigma-Aldrich, Poole, UK) were prepared as previously described and diluted four times with ACN before LC-MS analysis. Ammonium acetate was purchased from Sigma-Aldrich (Poole, UK).

4.5. Sample Preparation

Exactly 200 µL of the sample was mixed with 800 µL ACN containing 10 µg/mL of 2 ^{13}C glycine (Sigma-Aldrich) as an internal standard to ensure retention time stability, then centrifuged for 10 min before transferring into a vial with an insert. In order to prepare the QC sample, 0.05 mL of plasma was taken from each of the samples and mixed in order to make a pooled sample. The pooled sample was prepared by pipetting 50 µL from each of the 18 samples and then mixing them together before diluting 0.2 mL of the pooled sample with 0.8 mL ACN containing 5 µg/mL of 2 ^{13}C glycine internal standard and centrifuging. Additionally, the prepared mixtures of authentic standard metabolites containing 10 µg/mL of 2 ^{13}C glycine as internal standard were run.

4.6. LC-MS Conditions

Liquid chromatographic separation was carried out on an Accela HPLC system interfaced to an Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) using both a ZIC-pHILIC column (150 × 4.6 mm, 5 µm, HiChrom, Reading, UK). The column was eluted with a mobile phase consisting of 20 mM ammonium carbonate in HPLC-grade water (solvent A) and ACN (solvent B), at a flow rate of 0.3 mL/min. The elution gradient was an A:B ratio of 20:80 at 0 min, 80:20 at 30 min, 92:8 at 35 min and finally, 20:80 at 45 min. The nitrogen sheath and auxiliary gas flow rates were maintained at 50 and 17 arbitrary units. The electrospray ionisation (ESI) interface was operated in both positive and negative modes. The spray voltage was 4.5 kV for positive mode and 4.0 kV for negative mode, while the ion transfer capillary temperature was 275 °C. Full scan data were obtained in the mass-to-charge ratio (m/z) range of 75 to 1200 for both ionisation modes on the LC-MS system fully calibrated according to the manufacturer's guidelines. The resulting data were acquired using the XCalibur 2.1.0 software package (Thermo Fisher Scientific).

4.7. Data Extraction and Analysis

Patients were grouped into two and analysed based on percentage weight loss (> or <5%). Data extraction for each of the samples was carried out by MZMatch software. The extracted ions, with their corresponding m/z values and retention times, were pasted into an Excel macro of the most common metabolites prepared in-house to facilitate identification. The lists of the metabolites obtained from these searches were then carefully evaluated manually by considering the quality of their peaks and their retention time match with the standard metabolite mixtures run in the same sequence. All metabolites were within 3 ppm of their exact masses. The list of metabolites was refined by removing all metabolites with RSD > 20% in the pooled samples, leaving a list of 318 metabolites. Statistical analyses were performed using both univariate with Microsoft Excel and multivariate approaches using SIMCA-P software version 14.1 (Umetrics, Umea, Sweden). The 318 metabolites were modelled to give a PCA plot and then supervised analysis based on OPLS was carried out by

refining the list of metabolites by eliminating the less important variables to give a model with the lowest possible number of variables.

5. Conclusions

These results show that metabolomic profiles in plasma from cancer patients are different between patients with ≥ or <5% weight loss. Most of the metabolites identified within these profiles fell within the lipid pathways. Differences highlighted in the breakdown of lipids provide an understanding of the mechanisms involved in the pathogenesis of cachexia. A six-metabolite signature correlated strongly with degree of patient weight loss. A better understanding of the importance of adipose wasting and the potential sharing of datasets between studies may identify novel biomarker strategies and therapeutic approaches for cancer cachexia.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6694/11/10/1594/s1, Figure S1: PCA-X analysis.

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